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EFFECTS OF X-RAY IRRADIATION ON THE DEVELOPMENT OF OVARIAN TUMORS IN INTRASPLENIC GRAFTS IN CASTRATED MICE[1] [2]

By Min Hsin Li, Ph. D.,[3] W. U. Gardner, Ph. D., and Henry S. Kaplan, M. D.,[4] *Departments of Anatomy and Radiology, Yale University School of Medicine*

INTRODUCTION

The development of granulosa-cell tumors, luteomas, and tubular adenomas in the ovaries of mice after exposure to roentgen rays has been reported by Furth and Butterworth (*1*) and others (*2–6*). More recently, similar tumors have been observed in intrasplenic and intrapancreatic ovarian grafts in castrated rats (*7*) and mice (*8–10*).

This paper presents some results of experimental attempts to influence, by roentgen irradiation, the development of tumors in ovaries grafted into the spleens of castrated mice, and to detect, if possible, any synergistic action of these two tumorigenic stimuli. Four experiments were established to determine the effect of (1) roentgen irradiation directed to the spleens, or (2) directed to the pituitary glands of castrated mice bearing intrasplenic ovarian grafts; (3) irradiation in vitro of ovaries prior to grafting into the spleens of other castrated mice, and (4) grafting of both an in vitro irradiated ovary and an untreated ovary into the spleen in another series of castrated mice.

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[3] Present address, the University of Peking, Peiping, China.
[4] Now at the National Cancer Institute.

EXPERIMENTAL PROCEDURE AND RESULTS

Male and female mice of the A, C3H, C57 black, and JK strains, and several groups of hybrid mice (A×C3H and CBA×C57 black) were used. They were kept in an air-conditioned room and fed Purina Fox Chow and water. The four experimental groups and the number of ovarian tumors observed are summarized in table 1.

Vaginal smears of the castrated female mice were taken at intervals during the experiments. At autopsy, the ovarian grafts, genital tissues, livers, adrenal glands, kidneys, submaxillary glands, and pituitary glands were examined and preserved for histologic study. Some of the ovarian tumors which developed were transplanted into other mice of the same strains to test their capacity for continuous growth in another host.

Group 1: Irradiated, Intrasplenic Grafts

Eight male and nine female mice 34 to 69 days old were castrated, and an autologous or homologous ovary was grafted into the spleen of each. Eight to fifty-two days later, roentgen irradiation

Table 1.—*Effects of radiation on the development of tumors in intrasplenic ovarian grafts*

Group	Sex of host	Number of mice	Dose and method of irradiation	Ovarian tumors							
				Granulosa-cell		Luteoma		Mixed		None	
				Number	Age of graft	Number	Age of graft	Number	Age of graft	Number	Age of graft
					Days		*Days*		*Days*		*Days*
1	Male	8	400 r over graft	5	178–276	0		3	252–258	0	
	Female	9	do	2	208, 248	3	266–304	2	260, 283	2	183, 209
2	Male	6	400 r over the hypophysis	1	250	0		3	259–307	2	213, 229
	Female	6	do	3	248–275	1	237	1	282	1	203
3	Male	4	Graft given 200 r in vitro	0		0		1	266	3	137–178
	Female	7	do	1	204	1	150	2	266	3	83–211
4	Male	4	1 of 2 grafts given 200 r in vitro	0		1	[1] 251	1	[2] 252	2	[3] 160–190
	Female	2	do	0		1	[3] 250	0		1	[3] 182

[1] Irradiated graft. [2] Untreated graft. [3] Both grafts.

in a single dose of 400 r [5] was administered over the splenic area.

All eight male mice developed ovarian tumors, five of which were of the granulosa-cell variety, while three exhibited a mixture of granulosa and luteinlike cells. The ages of the tumorous grafts ranged from 178 to 276 days, and the largest tumor measured 5.5 x 12 x 14 mm. (fig. 1).

The nine female mice yielded seven ovarian tumors, three of which were luteomas, and two each were of the granulosa-cell or mixed varieties. All the tumors were discovered more than 200 days after grafting. The largest tumor measured 8 x 9.5 x 11 mm. Two mice had no tumor at the site of the graft 183 and 209 days, respectively, after operation; the small residual grafts consisted almost entirely of luteinized interstitial tissue.

The gross appearance of the tumors was similar to that described previously (*8, 9*). The tumors were much larger than the original grafts and protruded from the surface of the spleen, to which they were connected by broad stalklike structures. The splenic tissues were separated in part from the tumors by spaces lined by germinal epithelium, resembling the ovarian bursa.

The histologic characteristics of these tumors were in most respects similar to those previously described (*6, 9, 11, 12*). The granulosa-cell tumors were predominantly of the folliculoid variety although masses of tumor cells exhibiting a trabecular or diffuse arrangement were also observed. One mixed tumor in a female mouse was composed of spindle-shaped cells resembling those found in thecomas (*6*), intermingled with granulosa cells and giant cells (fig. 2).

The giant cells had large, hyperchromatic, often multilobular nuclei and prominent nucleoli and were scattered either singly or in small aggregates among the tumor cells and in the luteinized interstitial tissue (fig. 3).

Ingrowths from the germinal epithelium, small structures resembling anovular follicles, large cysts occasionally containing coagulated fluid and macrophages (fig. 4), and other familiar features (*3, 11, 12*) were also noted in many of the tumors. Direct contact of the tubular epithelial ingrowths and the tumor cells was noted in some areas (figs. 5, 6, 7) suggesting the transformation of the former into the latter (*9*).

In one luteoma there appeared to be a conversion

[5] In all experiments described, roentgen radiation was delivered with a superficial therapy apparatus at 100 kv. peak, 6 ma., inherent filtration, 17.8 cm. target-object distance, and an output of 200 r per minute, measured in air.

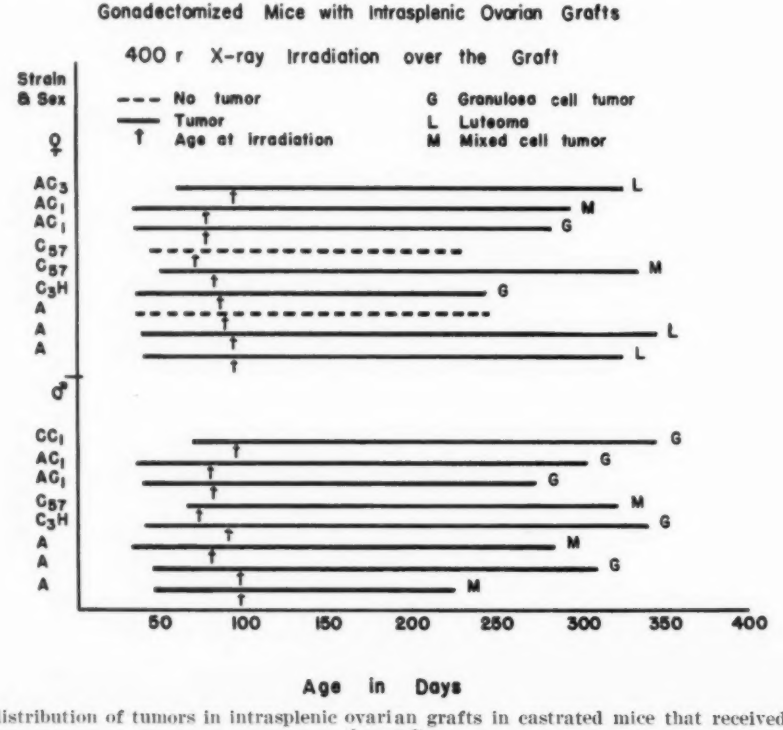


FIGURE 1.—Age distribution of tumors in intrasplenic ovarian grafts in castrated mice that received irradiation over the grafts.

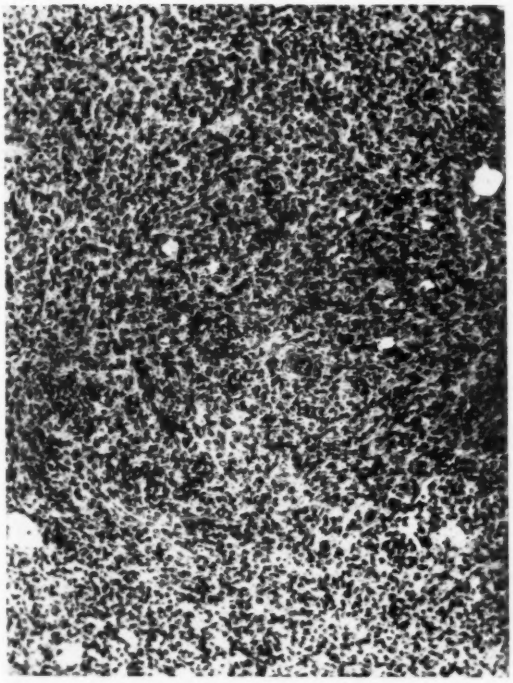


FIGURE 2.—Mixed tumor of folliculoma-thecoma type, containing giant cells, in an in vivo irradiated ovarian graft in a castrated female host. × 85.

of granulosalike cells into luteinlike tumor cells. A small spicule of bone was seen in a necrotic area within one of the granulosa-cell tumors. No normal ovarian follicles were observed in any of the tumors or in the two nontumorous grafts in this experimental group.

TABLE 2.—*Vaginal smear reactions and uterine weights of female mice that received roentgen irradiation (400 r) over their intrasplenic ovarian grafts*

Stock or strain	Ovarian tumor	Estrus vaginal smears	Uterine weight
			Milligrams
AC_3	Luteoma	None observed	40
AC_1	Mixed	Before and immediately after irradiation.	33
AC_1	Granulosa-cell	None observed	38
C57	None	Irregularly long after irradiation	24
C57	Mixed	About 30 days following irradiation.	79
C3H	Granulosa-cell	Before and immediately after irradiation.	103
A	None	do	35
A	Luteoma	do	18
A	do	do	32

The male mice had atrophic seminal vesicles and prostate glands, and narrow x-zones in the adrenal glands. All but two of the female mice exhibited cornified vaginal smears at some time during the experiment. The character of the vaginal smears and the uterine weights are presented in table 2. The histologic features of the other organs in both sexes did not differ from those previously reported (*13*).

GROUP 2: HYPOPHYSEAL IRRADIATION

Castration and intrasplenic ovarian grafting were performed on six male and six female mice at 32 to 67 days of age, as described for the first group. Seventeen to fifty-six days later, they were immobilized on their backs, and roentgen radiation, in a single arbitrarily selected dose of 400 r, was delivered to the hyperextended neck over the hypophyseal region. The remainder of the body was carefully shielded with lead as before.

One granulosa-cell tumor and three mixed tumors (fig. 8) developed intrasplenically 250 to 307 days after grafting in the six male mice. The largest tumor measured 6 x 7 x 14 mm. Two mice exhibited no tumors 213 and 229 days, respectively, after the implantation of ovarian grafts (fig. 9).

Among the females, one graft showed no neoplastic change after 293 days, while the remainder gave rise to three granulosa-cell tumors, one luteoma, and one mixed tumor after periods rang-

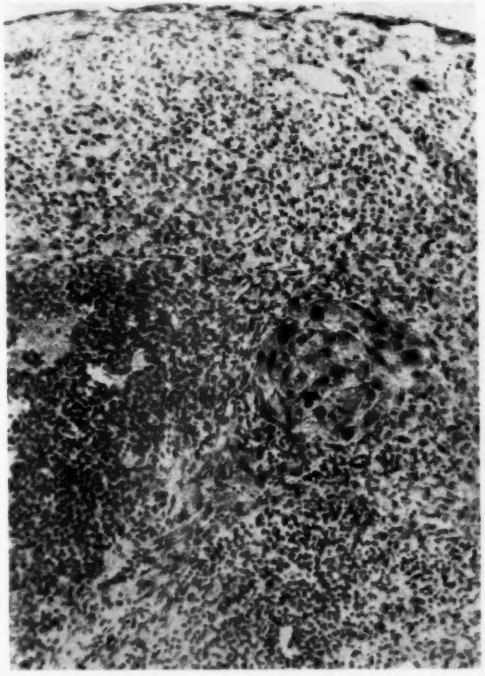


FIGURE 3.—Small aggregate of giant cells in the interstitial tissue adjacent to a folliculoid structure (left). × 85.

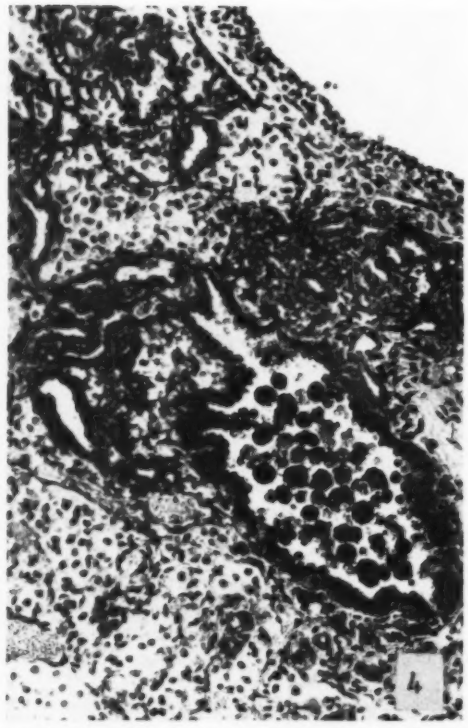


FIGURE 4.—Macrophages in the lumen of a tubule arising from the germinal epithelial ingrowth, lined by brown pigment-containing epithelial cells (250 days). ×170.

ing from 237 to 282 days. The largest tumor measured 18 x 23.5 x 30 mm.

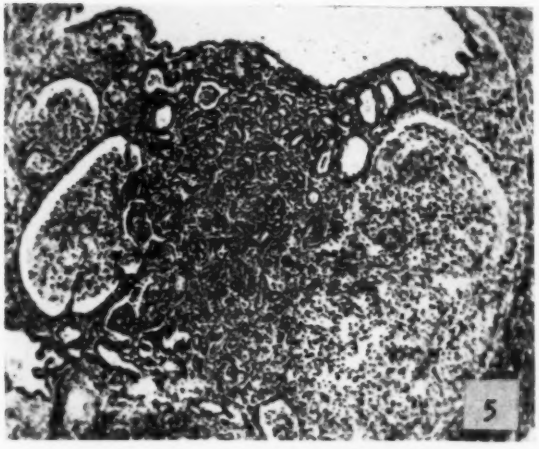
On gross and histologic examination, these tumors revealed no unusual features. In contrast with the previous group, however, normal ovarian follicles of various sizes and stages of development or regression were present in the grafts, usually near the periphery when tumors were present, in hosts of both sexes. The vaginal smear reactions and uterine weights of the females are summarized in table 3. The genital and other tissues of the males resembled those in group 1.

TABLE 3.—*Vaginal smear reactions and uterine weights of female mice bearing intrasplenic ovarian grafts and irradiated (400 r) over the hypophyseal region*

Stock or strain	Ovarian tumor	Estrus vaginal smears	Uterine weight
			Milligrams
AC_2	Granulosa-cell	Before and immediately after irradiation.	30
AC_2	do	Before irradiation	27
AC_1		None observed	30
C57	Mixed	Irregular	40
C3H	Granulosa-cell	About 40 days before autopsy	139
A	Luteoma	Before and immediately after irradiation.	50

No x-zone was observed in the adrenal glands of the female hosts, and their submaxillary glands and kidneys showed castration changes.

Small fragments of one granulosa-cell tumor from a strain C3H female mouse were transplanted either subcutaneously or intraperitoneally into several intact and castrated mice of the same strain, a 13-gage trocar being used. Progressive subcutaneous growths occurred in two male mice, one intact and the other castrated, and the abdominal cavity of another castrated male



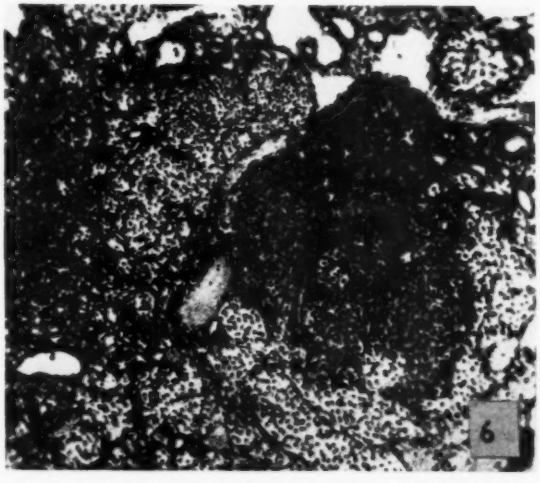


FIGURE 5 (upper).—Tubular adenomalike and glomerulus-like growths from the germinal epithelium in a non-tumorous graft irradiated in vitro prior to intrasplenic implantation 137 days earlier in a castrated male host. ×85.

FIGURE 6 (lower).—Small group of granulosa cells surrounding tubular structures of the germinal epithelial ingrowth in an intrasplenic ovarian graft (182 days) in a castrated female mouse. ×85.

killed 100 days after inoculation contained a tumor mass 7 x 8 x 15 mm. in size which histologically resembled the original tumor.

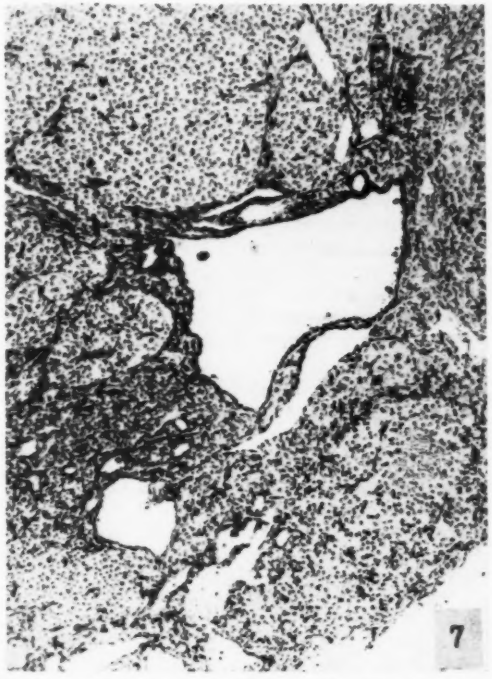


FIGURE 7.—Tubular ingrowths closely associated with tumor cells of a luteoma which developed in an in vivo irradiated intrasplenic ovarian graft in a castrated female mouse (304 days). ×85.

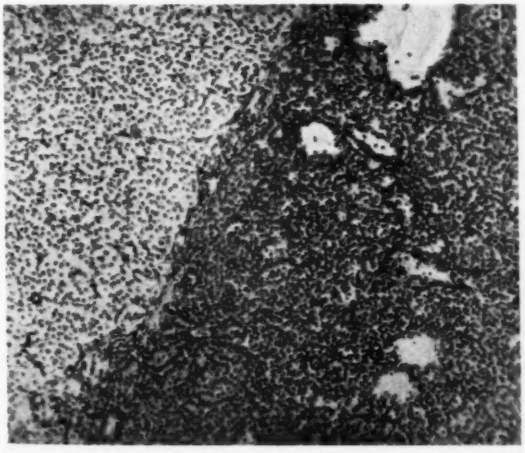


FIGURE 8.—Mixed tumor of folliculoma-luteoma type 307 days after intrasplenic implantation of an ovary into a castrated male host irradiated over the hypophysis. ×85.

GROUP 3: GRAFTS IRRADIATED IN VITRO

Four male and seven female mice 23 to 30 days old were subjected to gonadectomy and the implantation of a pre-irradiated autologous or homologous ovary into the spleen. A single dose of 200 r, measured in air, was delivered to the excised ovaries which had been placed on filter paper, moistened with physiologic saline solution, in sterile petri dishes. Only five of the animals developed tumors at the site of grafting, the distribution and latent period of which are indicated in table 1. In general these tumors were smaller than those noted in animals of groups 1 and 2.

Except for the occasional presence of bone spicules (fig. 10), these tumors exhibited no remarkable histologic features. The genital tissues and other organs resembled those of animals in the other groups. One female host in which a granulose-cell tumor developed showed cornified epithelial cells in its vaginal smears for some time prior to autopsy and was found to have a uterine weight of 80 mg. Of the remaining six females, one tumor-free individual showed irregular estrus vaginal smears during the early part of the experiment, while the other five showed none at any time. The average uterine weight of these six females was 20.8 mg.

GROUP 4: DOUBLE IMPLANTATION WITH IRRADIATED AND NONIRRADIATED OVARY

Two autologous or homologous ovaries were implanted in each of six gonadectomized mice (four male, two female) at 70 to 77 days of age. One of the ovaries was implanted intrasplenically directly after excision and received no irradiation. The other ovary was exposed in vitro, as described for group 3, to a single X-ray dose of 200 r, and then implanted either proximal or distal to the first, with an average interval of 3 to 4 hours between excision and inoculation.

As indicated in table 1, one of the female mice developed luteomas in both of its ovarian grafts. No tumors appeared in either graft in the other female, or in two of the males. The other two males exhibited, respectively, a luteoma in the irradiated graft only, and a mixed tumor in the untreated graft alone. In addition, areas of granulosa-cell proliferation of suggestively pretumorous character were observed in two nonirradiated grafts. Ovarian follicles were not observed in any of the irradiated grafts but were present in both the tumors and nontumorous grafts that had not been exposed to radiation.

DISCUSSION

Roentgen rays destroy ovocytes and prevent the formation of normal ovarian follicles in mice (*14–17*). That ovaries of irradiated mice produce

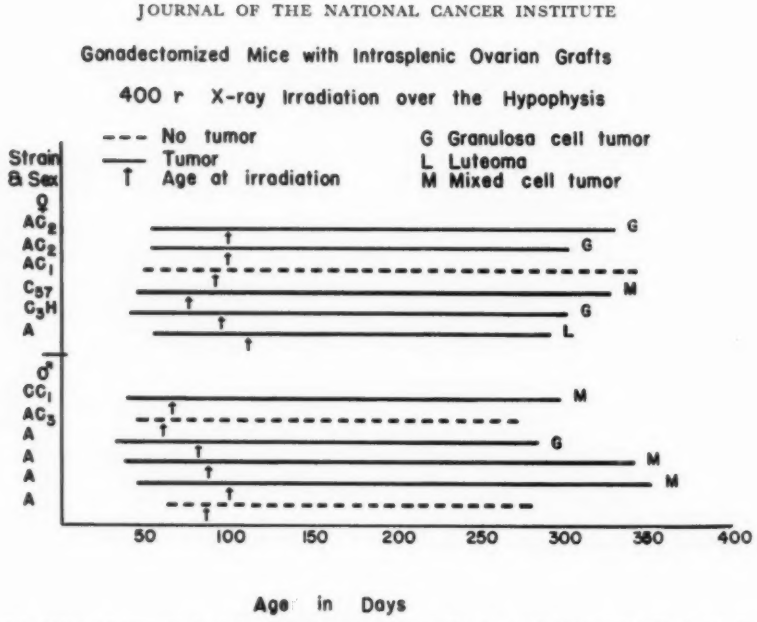


FIGURE 9.—Age distribution of tumors in intrasplenic ovarian grafts in castrated mice that received irradiation over the hypophysis.

reduced amounts of estrogen is suggested by the fact that estrus cycles are irregular and usually prolonged (*12*). Some time after irradiation, there is an ingrowth of cords of cells from the germinal epithelium; and many treated ovaries ultimately give rise to granulosa-cell tumors or luteomas if the irradiated mice survive for approximately a year or more (*1, 3, 4, 12*).

Such tumors also occur regularly in castrated mice surviving 150 days or more after the intrasplenic implantation of ovarian grafts (*8–10*). Such grafts are believed to become neoplastic as a result of their exposure over a prolonged period to an abnormal environment characterized by an excess of hypophyseal gonadotrophic hormones, probably chiefly of the follicle-stimulating type. In view of the decreased frequency or complete cessation of estrus in irradiated female mice (*12, 15*), it seems likely that radiation causes a diminution of estrogen secretion which in turn might be expected to result in an overproduction of gonadotrophic hormones by the hypophysis. It is, therefore, at least theoretically possible that roentgen radiation acts by the same mechanism as has been postulated for intrasplenic implantation in the induction of such ovarian tumors. It was partially to test this hypothesis that these experiments were performed.

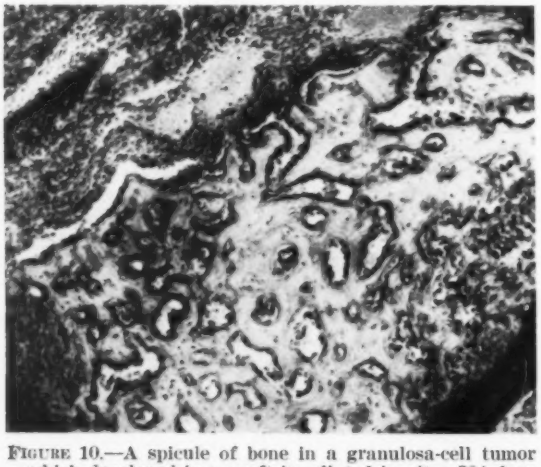


FIGURE 10.—A spicule of bone in a granulosa-cell tumor which developed in a graft irradiated in vitro 204 days earlier. × 170.

It was hoped that the demonstration of synergism of these two tumorigenic stimuli, roentgen rays and intrasplenic implantation, or the failure to demonstrate such synergism would shed light on the mechanism by which radiation acts in eliciting ovarian tumors in mice. The results indicate no synergistic effect as compared with the previously reported incidence and induction time in nonirradiated, intrasplenic ovarian grafts. Unfortunately, no definite conclusions can be drawn from these data. Untreated intrasplenic ovarian grafts in castrated mice become neoplastic with an incidence approximating 100 percent, after latent periods of 5 to 10 months. It may well be

that this is already a maximal stimulus for ovarian neoplasia of this type and that no further increase in incidence or reduction in latent period can be expected from exposure to an additional tumorigenic agent.

Most of the published investigations dealing with the induction of ovarian tumors by irradiation do not contribute to a solution of this problem because whole-body exposures of the animals to rather large doses were employed. Mice of the A strain were irradiated with fractional doses totaling 2,100 r over 1 of 4 small body areas (head, chest, lumbar region, and base of tail), with the result that 5 of 15 surviving females treated over the lumbar area, and therefore also directly over the intact ovaries, developed tumors while none of the females in the other groups did so.[6] This result would indicate that the radiation must act directly upon the ovary to elicit these neoplasms, but the possibility is still not excluded that this initial effect of radiation is simply a depression of estrogen secretion, which in turn is followed by overproduction of pituitary gonadotrophic hormones and ultimately by ovarian-tumor induction.

Lorenz and his co-workers (*5*) exposed F_1 hybrid mice of strain A male × strain L female parentage to chronic low-intensity gamma radiation in doses of 0.11, 1.1, 2.2, 4.4, or 8.8 r per 8-hour day, and found that the incidence of ovarian tumors was largely independent of the dose rate. Moreover, in collateral experiments, they gave similar mice single 5-hour roentgen ray exposures of only 50 r and succeeded in inducing tumors in over two-thirds of the animals. Furth (*2*) also observed luteomas, granulosa-cell tumors, or tubular adenomas in 31 of 44 mice with single whole-body doses of only 87 r. There are few data available concerning the effect of such low doses upon estrogen secretion, but it is probable that it is but little, if at all, affected by such doses since they are insufficient to destroy many of the ovocytes or follicles and do not produce sterility. If it can be established that estrogen is not depleted by such small amounts of radiation, the only tenable conclusion would be that radiation acts directly upon the ovary in producing these neoplasms rather than by the indirect mechanism involved in intrasplenic implantation.

The irradiated and nonirradiated ovarian grafts differed in one respect: ovocytes and ovarian follicles were destroyed by irradiation. Nevertheless, tumors occurred with a comparable frequency in both groups. The presence of small ovarian follicles and such hormones as they might produce did not alter the incidence or the time required for the development of these tumors. The previously reported observation of a preponderance of luteomas or mixed tumors in female hosts and of granulosa-cell tumors in male hosts (*8*) was again noted in the irradiated intrasplenic grafts. These observations suggest that the ovarian tumors arise from ovarian tissue derived from the germinal epithelium rather than from pre-existing granulosa cells of ovarian follicles.

In view of the possibility that ovarian tumorigenesis is attributable to an abnormal humoral environment high in gonadotrophic hormones, it was of interest to study the effect of irradiation of the hypophysis and incidently of adjacent tissues. The dose of roentgen rays (400 r) administered to the hypophyseal area was probably not sufficient to suppress the activity of the pituitary gland to an appreciable extent. The low radiosensitivity of the pituitary gland has been reported by several investigators (*18; 19, p. 55*), who noted no permanent inhibition of hypophyseal function in rats and other animals after much larger doses. The presence of ovarian follicles in the grafts was not visibly affected by pituitary irradiation as administered in this experiment.

The formation of bone in ovarian tumors is not a specific effect of roentgen irradiation because osseous spicules have been observed in tumors that arose in intrasplenic ovarian grafts in castrated mice treated with progesterone and pregnant mare's serum (*20*), in subcutaneously transplanted granulosa-cell tumors (*13*), and in a granulosa-cell tumor that developed in an intrapancreatic ovarian graft in a castrated mouse (*21*). Although osteogenesis has been described in ovarian tumors (*22, p. 138*), little is known of the mechanisms involved. Alkaline phosphatase is abundant in the cells of the theca interna in some species and in the granulosa cells of others (*23*). Although its function in the ovary is not clear, the possibility exists that its presence may be related to the tendency for tumors of the ovary to form osseous tissue.

The mixed tumors of folliculoma-thecoma type containing giant cells were not observed among the ovarian tumors described previously from this laboratory, but similar cells have since been observed in ovarian tumors from mice used in another group of experiments.[7] These giant cells differ from the luteallike giant cells described by Brambell, Parkes, and Fielding (*15*).

Unlike tumors of some organs, ovarian tumors in intrasplenic grafts in castrated mice have so far revealed no strain limitation. In these experiments, they occurred in mice of the JK strain, as well as in mice of the C3H, C57, and A strains and in several hybrid groups in which they had been observed previously (*20*).

Both the irradiated and the nonirradiated ovarian grafts that were not completely occupied by tumor cells showed a close association of the small tumorous areas with cells derived from the

[6] KAPLAN, H. S.: Unpublished observations.

[7] LI, M. H., and GARDNER, W. U.: Unpublished observations.

germinal epithelium. Such ingrowths occurred in grafted ovaries that did not become tumorous and have been observed in ovaries grafted at sites other than the spleen, i. e., the testis, where ovarian tumors do not form (*9*). It is considered that prolonged stimulation by augmented gonadotrophic hormones contributes to neoplastic change of cells derived from the germinal epithelial ingrowth of intrasplenic or intrapancreatic ovarian grafts in castrated mice.

SUMMARY AND CONCLUSIONS

Granulosa-cell tumors, luteomas, or mixed tumors developed in irradiated (400 r), intrasplenic ovarian grafts in seven of nine castrated female mice and in all of eight castrated male mice. The incidence and time of origin of these tumors were not modified by irradiation. Neither ova nor normal ovarian follicles were observed in any of the irradiated grafts.

Roentgen irradiation of the hypophysis and adjacent tissues (400 r) did not inhibit the formation of the intrasplenic ovarian tumors which developed in 9 of 12 mice so treated.

Ovaries irradiated in vitro (200 r) and subsequently grafted into the spleens of castrated hosts gave rise to tumors but with a lower incidence than did grafts irradiated in vivo.

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EVALUATION OF THE CARCINOGENICITY OF A SERIES OF ESTERS OF CARBAMIC ACID[1,2]

By C. D. LARSEN, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Nettleship and Henshaw (*1*) reported striking increases in the incidence and multiplicity of pulmonary tumors in strain C3H and strain A mice following repeated narcotization with ethyl carbamate (urethane). Subsequently (*2, 3*), it was noted that the carcinogenic effect was exerted on pulmonary tissue only, and that comparable tumor multiplicities were obtained when the route of administration was intraperitoneal, subcutaneous, or oral. Of a variety of narcotizing agents examined, only urethane was found to be effective (*3*).

Preliminary to further study of the mechanism of action of ethyl carbamate, a number of other esters of carbamic acid have been studied with respect to carcinogenic activity for pulmonary tissue of the mouse. The results are reported herein.

EXPERIMENTAL PROCEDURE AND RESULTS

Strain A mice, because of their greater susceptibility to lung-tumor induction, were used in each experiment. In previous investigations of the effects of urethane on strain A mice a sex difference in susceptibility was not observed.[3] Hence all groups reported herein contained approximately equal numbers of each sex. All mice were maintained on the National Cancer Institute stock diet (*4*) and water. Healthy mice, 10 to 12 weeks of age, were injected intraperitoneally with a solution of the compound under examination. Injections were given once a week for 13 weeks. Two to three weeks after the final injection, when the mice were 6 months of age, they were killed and examined in the manner previously described (*3, 5*). Differences in technique, from one experiment to another, owing to tolerance and solubility phenomena, are noted herein.

METHYL CARBAMATE

Synthesis of methyl carbamate was effected by the interaction of methyl chlorocarbonate and ammonia. The product was isolated and purified by recrystallization from ether; melting point, 56°–57° C.[4] Forty-six mice were injected intraperitoneally with 0.01 ml. per gram mouse of a 5-percent solution of the ester in water. No perceptible narcosis or evidence of toxicity was observed. The mice were killed and examined 2 to 3 weeks after the final injection. Neither the lung-tumor incidence, 16 percent, nor the multiplicity of tumors, average 0.19 per mouse, differed from corresponding data for control mice (table 1). In other groups of 43 and 49 mice, injected weekly with 2 and 4 times the foregoing dose (1.0 and 2.0 mg. per gram mouse), tumor incidences of 9 and 22 percent and averages of 0.09 and 0.29 tumors per mouse, respectively, were observed. Random samples of the control group exhibited variations to the same extent. Only slight narcosis was observed with the 2.0-mg. dose. Administration of 3.0 mg. per gram mouse resulted in moderate anesthesia in 5 minutes; 2 of 5 mice died within 48 hours. A dose of 4.0 mg. killed all of 5 mice within 48 hours.

TABLE 1.—*Pulmonary-tumor induction by esters of carbamic acid*

Compound[1]	Dose[2] (milligram per gram mouse)	Number of mice	Mice with tumors	Mean number of tumors	Relative narcosis
			Percent		
Methyl	0.5	46	16	0.19	None.
Ethyl	.5	102	100	76.9	Moderate.
n-Propyl	.5	78	63	.95	Deep.
Isopropyl	.5	51	90	3.7	Moderate to deep.
n-Butyl	.25	33	12	.12	Do.
Isoamyl	.5	23	13	.13	Deep.
Chloroethyl	.5	46	15	.15	Shallow.
Trichloroethyl	.25	53	42	.54	Moderate to deep.
Control		141	17	.18	

[1] Esters of carbamic acid.
[2] Intraperitoneal injection, weekly for 13 weeks.

ETHYL CARBAMATE

Urethane of commercial quality was recrystallized and administered at a level of 0.5 mg. per gram mouse, which was followed by relatively shallow anesthesia. Following 13 weekly injections, lung tumors were found in all of 102 mice; average tumors per mouse, 76.9.[5] Variation in response, from mouse to mouse, is shown in table 2.

TABLE 2.—*Variations in number of pulmonary tumors induced with urethane*[1,2]

Number of mice	Mice showing given number of tumors per individual										
	1–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100	101–110	111–120
102	4	0	0	2	5	6	14	15	29	17	9	1

[1] 0.5 mg. per gram mouse, weekly for 13 weeks.
[2] Strain A.

[5] In a previous experiment (*3*), an average of 89.2 was observed.

[1] Received for publication November 24, 1947.
[2] Presented in part at the meetings of the American Association for Cancer Research, Chicago, May 15–17, 1947.
[3] Unpublished data.
[4] All melting points are corrected temperatures.

n-Propyl Carbamate

Thirty-two mice were injected weekly with 0.5 mg. per gram mouse of n-propyl carbamate as obtained from the manufacturer (Eastman Kodak Co.). This dose induced deep narcosis within 3 minutes. Two weeks after the thirteenth injection a lung-tumor incidence of 59.5 percent was observed in 32 mice; the average tumor count was 0.9 per mouse. Owing to moderate increases in these values when compared with those for control mice, the possibility of a trace contamination with ethyl carbamate suggested itself. Accordingly, a sufficient quantity of the commercial material was repeatedly recrystallized from water, melting point 61°–62° C. Calculated for $C_4H_9O_2N$ (propyl): N=13.6 percent; for $C_3H_7O_2N$ (ethyl): N=14.1 percent; found: N=13.4 percent.[6] A group of 46 mice, treated with the purified material in the previously described manner, showed a tumor incidence of 65 percent and an average of 1.0 tumor per mouse, values essentially identical with those obtained in the mice injected with the commercial grade. Data for these two groups were combined for tabulation in table 1. These tumors, each of which was confirmed microscopically, were strikingly small; on the average they appeared to be roughly one-third the size of the usual pulmonary adenomas in strain A mice of this age. Variation within the group is shown in table 3.

Isopropyl Carbamate

Synthesis was achieved by reaction of isopropyl chlorocarbonate [7] with ammonia. The isolated product was crystallized from a mixture of ethyl ether and petroleum ether (1:3); melting point 94.0°–94.4° C. Intraperitoneal injection of 0.5 mg. per gram mouse (5-percent solution in water) was well tolerated. Narcosis developed less rapidly and was less profound and of shorter duration than that obtained with the isomeric ester. After the usual series of injections, it was found that 46 of 51 mice (90 percent) had developed 1 or more pulmonary tumors; an average of 3.7 tumors per mouse was observed. The degree of variation in response is shown in table 3.

n-Butyl Carbamate [8]

This ester, because of low solubility in water, was dissolved in pure tricaprylin (2.5 gm. per 100 ml.). Dosages of 0.5 or 0.375 mg. of the ester per gram mouse induced a rapid and deep narcosis, but about 40 percent of the mice died within 2 hours. A dosage of 0.25 mg. induced a somewhat lesser degree of anesthesia, and all of 33 mice survived the course of 13 weekly injections. One lung tumor was found in each of 4 mice (12 percent incidence), and the number of tumors per mouse averaged 0.12.

Isoamyl Carbamate [9]

Twenty-three of forty-one mice survived 13 weekly injections of 0.5 mg. isoamyl carbamate per gram mouse (5-percent solution in tricaprylin). Deep anesthesia resulted in about 3 minutes. No increase in incidence or multiplicity of lung tumors was noted; one tumor was observed in each of three mice.

β, β, β-Trichloroethyl Carbamate [10]

This halogenated urethane was recrystallized from ethyl alcohol; melting point 63°–64° C. Calculated for $C_3H_4O_2NCl_3$: N=7.3 percent, Cl=55.3 percent; found: N=7.1 percent, Cl=55.4 percent. Moderate to deep narcosis developed within 15 minutes following administration of 0.25 mg. per gram mouse (5-percent solution in tricaprylin). Twice this dose produced deep anesthesia in less than 10 minutes but proved fatal to half the mice. The tolerated dose (0.25 mg.) was injected weekly for 13 weeks. At autopsy, the observed pulmonary-tumor incidence was 42 percent, and the average number of tumors per mouse was 0.54. Variation in response, from mouse to mouse, is noted in table 3.

β-Chloroethyl Carbamate

Synthesis was effected by the interaction of β-chloroethyl chlorocarbonate and ammonia. The

[6] All elementary analyses were performed by C. A. Kinser.
[7] Kindly furnished by Columbia Chemicals Division, Pittsburgh Plate Glass Co.
[8] Kindly furnished by Sharples Chemicals Inc.
[9] Furnished by Sharples Chemicals Inc.
[10] Kindly furnished by Dr. L. C. Miller, Winthrop Chemical Co., Inc.

TABLE 3.—*Variations in number of pulmonary tumors induced with carbamates of low activity*

Compound	Number of mice	Mice [1] showing given number of tumors per individual									Mice with tumors	Mean number of tumors
		0	1	2	3	4	5	6	7	8		
											Percent	
Isopropyl carbamate [2]	51	5	4	8	8	9	3	7	4	3	90	3.7
n-Propyl carbamate [2]	78	29	32	14	2	.....	.....	.....	.....	1	63	.95
Trichloroethyl carbamate [3]	53	31	18	3	1	.....	.....	.....	.....	.....	42	.54
Controls	141	117	23	1	.....	.....	.....	.....	.....	.....	17	.18

[1] Strain A.
[2] 0.5 mg. per gram mouse; weekly for 13 weeks.
[3] 0.25 mg. per gram mouse; weekly for 13 weeks.

product was isolated and recrystallized from 25 percent alcohol; melting point, 73°–74° C. Calculated for $C_3H_6O_2NCl$: N=11.3 percent, Cl=28.7 percent; found: N=11.1 percent, Cl=28.4 percent. Deep and prolonged narcosis developed within 3 minutes following intraperitoneal injection of 1.0 mg. per gram mouse (5-percent solution in water). Half of the mice given this dose died during the third and fourth days. Half of this dose, 0.5 mg. per gram mouse, produced only shallow anesthesia and was well tolerated for the course of 13 weekly injections. In a group of 46 mice, the observed lung-tumor incidence was 15 percent, and the number of tumors per mouse averaged 0.15. These values were no greater than in control mice.

DISCUSSION

The data derived from the several experiments are given in table 1. In the series of esters examined, it is evident that ethyl carbamate was unique in its ability to induce pulmonary tumors in strain A mice. In this series of experiments, urethane was the only compound which produced tumors in all mice. The relative potency of this compound is more effectively demonstrated by the observation that the average number of tumors in urethane-injected mice was 21 and 81 times greater than the means in the groups treated with the isomeric propyl esters. In decreasing order, isopropl, n-propyl, and trichloroethyl carbamate provided evidence of activity, although of an order much below that of urethane, when compared with data for control mice.

The data for all groups of mice were subjected to statistical analysis. The χ^2 and P values, demonstrating degrees of significance of differences in the proportion of mice showing tumors, and the t and P values, for the differences in the mean number of tumors are shown for several groups in table 4. These data demonstrate that the potency of isopropyl carbamate was significantly greater than those of the isomeric n-propyl ester and the trichloroethyl ester. Although the activity of n-propyl carbamate appeared to be greater than that of the trichloroethyl ester, it should be pointed out that the former was administered gravimetrically in twice as large a dose, and 3.8 times as large a dose on a molar basis. With equimolar quantities, it is possible that the potency differences between n-propyl and trichloroethyl carbamate might have been reduced or even reversed.

TABLE 4.—*Statistical analyses of differences in the incidence and mean number of pulmonary tumors* [1]

Compounds compared	Analyses of incidence differences		Analyses of mean differences	
	χ^2	P	t	P
Urethane vs. isopropyl	10.3	<0.001	23.4	<0.001
Urethane vs. n-propyl	45.2	<.001	30.1	<.001
Urethane vs. trichloroethyl	74.6	<.001	25.0	<.001
Urethane vs. controls	163.2	<.001	41.3	<.001
Controls vs. isopropyl	86.6	<.001	17.3	<.001
Controls vs. n-propyl	47.4	<.001	7.2	<.001
Controls vs. trichloroethyl	12.8	<.001	4.1	<.001
Isopropyl vs. n-propyl	11.9	<.001	8.9	<.001
Isopropyl vs. trichloroethyl	27.2	<.001	9.4	<.001
n-Propyl vs. trichloroethyl	5.8	<.01–.02	2.3	<.03

[1] χ^2 and P (probability of chance variation) have their usual statistical significance; t is the ratio of the observed difference to the standard error of the difference.

In support of earlier observations (*3*) the data presented herein demonstrate that the ability of a compound to elicit lung tumors in strain A mice is not associated with its capacity to induce narcosis.

SUMMARY

Relative potencies of the ethyl, isopropyl, and n-propyl esters of carbamic acid, as determined by the mean number of pulmonary tumors which they elicited under comparable conditions, were found to be of the order of 81, 4, and 1.

Trichloroethyl carbamate provided evidence of a significant, but low order of carcinogenicity, the exact evaluation of which was prevented by the relative toxicity of the compound.

The methyl, monochloroethyl, n-butyl, and isoamyl carbamates were inactive at the dosage levels employed.

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(*3*) LARSEN, C. D.: Survey of hypnotics in general with regard to pulmonary-tumor induction in mice. J. Nat. Cancer Inst. 7: 5–8 (1946).

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THE USE OF PERFORATED CELLOPHANE FOR THE GROWTH OF CELLS IN TISSUE CULTURE [1]

By VIRGINIA J. EVANS, *cytologist*, and WILTON R. EARLE, *principal cytologist*, with the technical assistance of EDWARD L. SCHILLING, *technologist*, ELIZABETH P. WILSON, *junior cytologist*, and EMILY DUCHESNE, *junior cytologist*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

For the satisfactory maintenance of cultures of most tissue cells in vitro, a solid support for the migration and growth of the cells is necessary. Both in this laboratory and in others, various types of supports have been used, such as silk thread, spider web, glass wool, lens tissue, gelatin, agar, the glass surface of the culture dish, cellophane, and clots made by the action of thrombin on fibrinogen or plasma. Plasma or fibrin clots have proved best for the routine maintenance of tissue cultures and are now in general use.

There are, however, many objections to the use of the plasma clot. Some of these are: (1) The preparation of the plasma or fibrinogen presents substantial technical difficulties; (2) under many culture conditions, the clot clouds and obscures cell detail, or liquefies and so destroys the culture, or impairs the accuracy with which it can be used; (3) the fact that the culture is embedded within the clot and cannot be removed from it interferes with determinations of the growth, weight, or chemical changes of the cultures, as well as the study of fixed and stained preparations; and (4) the chemically undefined nature of the clot interferes with studies of the metabolism of cells in cultures.

MATERIALS AND METHODS

Recently, through the courtesy of Dr. C. L. Blair, of the Technical Service Laboratory, Cellophane Division of the E. I. Dupont de Nemours & Co., Inc., a quantity of Dupont MSAT 82, thickness 600, cellophane, perforated with holes one thirty-second inch diameter on three thirty-second inch centers was made available for study. As a control unperforated cellophane Dupont PT 62, thickness 450, was used. Comparative studies were made of a number of strains of normal and sarcomatous fibroblasts when grown both on and under these two types of cellophane. The growth under these conditions was compared with that of cultures grown directly on the glass surface of the culture flask and also in the usual chicken-plasma clot. The early findings focused attention on growth under the perforated cellophane.

Modified Carrel-D flasks 3.5 or 5.0 cm. in diameter were used as culture vessels. The cellophane was cut into circles to fit the floor of the flask, after allowing for shrinkage in autoclaving, and was then washed in repeated changes of acetone, alcohol, ether, and triple-distilled water. The cellophane was air-dried, and one disk was inserted into each flask. The flasks were then autoclaved.

With D 3.5 flask cultures, the fluid culture medium (1.0 ml. of a mixture of 40 percent horse serum, 20 percent embryo-juice extract 1 : 1, and 40 percent Earle's saline [2]) was added to each flask and allowed to soak the cellophane for 1 to 3 minutes. The cell clumps were added by means of a platinum spatula and positioned on or under the cellophane sheet. The flask was then sealed and incubated as usual.

In cultures where the cell clump was allowed to grow directly on the glass floor of the flask, the clump was positioned in the flask and 1 ml. of the fluid culture medium added. In plasma-clot cultures in D 3.5 flasks the substrate consisted of 0.6 ml. of chicken plasma plus 0.8 ml. of the culture medium. This was overlaid with 1 ml. of the fluid culture medium.

In D 5.0 flasks this proportion was maintained, but the total volume of solid medium was increased to 2.8 ml. Where 5-cm. culture flasks were used, the culture fluid was increased to 2 ml. Late in the growth of the culture, the supernatant fluid was further increased to furnish food to the large mass of cells.

All cultures were washed with 2 ml. of saline, and fresh fluid culture medium was added three times weekly. Most cultures were carried for about 30 days. Seven groups of cultures were studied.

RESULTS

First Series, Mouse Sarcoma

The first group of cultures studied was of strain L, Earle's mouse sarcoma cells which had been grown in vitro since October 1940. Cultures of this strain were planted from plasma matrix cultures, under both perforated and unperforated cellophane.

At 5 days, migration in both sets was extremely scant. At 12 days, the cultures of both sets had reached a size comparable with that in the plasma-matrix cultures. At 19 days, they had reached the edge of the 3.5-cm. flasks and were so dense that some diffuse necrosis from cell crowding had set in. Growth under the perforated cellophane was somewhat more dense and showed less diffuse necrosis than under the unperforated cellophane.

At 19 days, fragments scraped from cultures were transferred to plasma cultures and also under

[1] Received for publication October 13, 1947.

[2] EARLE, W. R.: Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4 : 165–212 (1943).

perforated cellophane.[3] At 13 days, in the second generation there was less necrosis and better growth under the perforated cellophane than in the plasma. At 27 days, all cellophane cultures showed very heavy, packed growth extending to the edge of the flasks. There seemed no doubt that in this series of two transfer generations there was more luxuriant growth and less necrosis under the perforated than under the unperforated cellophane.

Second Series, Chick-Embryo Heart

In the second series fragments from 10-day-old chick-embryo hearts were planted in plasma, on glass, under unperforated, and on and under perforated cellophane. Here, too, migration for the first several days was better in the plasma cultures.

At 7 days (fig. 1, *A*), the width of the cultures in the plasma averaged about 14 mm., but cell density was rather low. Growth on the glass (fig. 1, *B*) was scant or absent. Under unperforated cellophane (fig. 2, *A*) growth was very irregular; in some cases, extremely scant; in others, luxuriant (fig. 2, *B*).

On perforated cellophane (fig. 3, *A*), the culture width averaged about 8 mm., but the cell density was greater than in the plasma. The cultures under the perforated cellophane (fig. 3, *B*) were in general somewhat better, averaging about 9 mm., with excellent cell density.

Close examination showed that in cultures grown on the cellophane, the cells had gone through the holes to the lower surface of the sheet. There appeared to be relatively few cells on the glass surface of the culture flask.

At 19 days, the cultures in plasma were satisfactory but not luxuriant; the cultures on glass had been discarded as hopeless. Of six cultures under unperforated cellophane, two were excellent, the cell sheet having grown to the edge of the cellophane, but four were largely necrotic. Of five cultures on perforated cellophane, all showed severe necrosis, particularly around the cellophane hole areas. Cultures under the perforated cellophane had reached the edge of the cellophane sheet, were luxuriant, and showed only slight diffuse necrosis, apparently due to cell crowding.

Third and Fourth Series, Mouse Fibroblasts

In the third and fourth series, the cultures studied were fresh mouse fibroblasts, planted as strips of ventral musculature from young adult C3H strain mice. These cultures were planted in plasma, on glass, on and under unperforated cellophane, and on and under perforated cellophane.

At 17 days, the plasma cultures (fig. 4, *A*) showed a zone of outgrowth only several millimeters wide, and even this was of moderately limited cell density. The growth of the cultures on glass (fig. 4, *B*) was even more scant; some showed complete necrosis. On unperforated cellophane the growth was scant or unsatisfactory because the cultures floated free from the cellophane. Some cultures under unperforated cellophane (fig. 5, *A*) showed a substantially better area of growth than did the plasma cultures, but growth was irregular; other cultures were almost entirely necrotic. Cultures on perforated cellophane (fig. 5, *B*) showed dense growth to the edge of the cellophane sheet, but here again it was often apparent that the cells had gone through the holes and were adhering to the lower surface of the cellophane disk (fig. 6, *A*).

Cultures under perforated cellophane (fig. 6, *B*) showed luxuriant, dense cell sheets reaching the edge of the cellophane, with relatively few cells adhering to the glass.

Fifth and Sixth Series, Mouse Fibroblasts and Mouse Strain H Sarcoma

In the fifth series, a strain of normal subcutaneous mouse fibroblasts carried in vitro more than 3 years was used. In the sixth series, cells of tissue cultures of the strain H of Earle's sarcoma were used. Both series were unsatisfactory because the cellophane disks were not cut oversize to allow for shrinkage in autoclaving, with the result that they floated free from the glass, and nearly all cultures were ruined. The results showed that for satisfactory growth the disks must be cut large enough to avoid slipping around over the surface of the flask. It was, however, also shown (fig. 7, *A*) that growth of strain H sarcoma cells under perforated cellophane was practicable.

Seventh and Eighth Series, Mouse Fibroblasts and Mouse Strain L Sarcoma

The seventh series was started from plasma matrix cultures of the same 3-year-old strain of normal-adult-mouse fibroblasts. The eighth series was started from plasma matrix cultures of Earle's L strain sarcoma. The two series were carried in parallel and are considered together. Cultures were grown in 3.5- and 5.0-cm. culture flasks.

Cellophane cultures showed slow migration of cells the first few days. At 19 days both normal (fig. 7, *B*), and malignant cultures (fig. 8, *A*) were growing nicely in plasma. Under unperforated cellophane (fig. 8, *B*), growth was very poor with much necrosis in the normal cultures. The L strain sarcoma (fig. 9, *A*) showed a cellular diffusion all over the surface of the cellophane with no patchy necrosis. Under perforated cellophane the normal cultures (fig. 9, *B*) were growing but were patchy. The cell sheets had pulled loose from the cellophane in extensive areas, had

[3] This study was made by Dr. Katherine Sanford, of this tissue-culture unit.

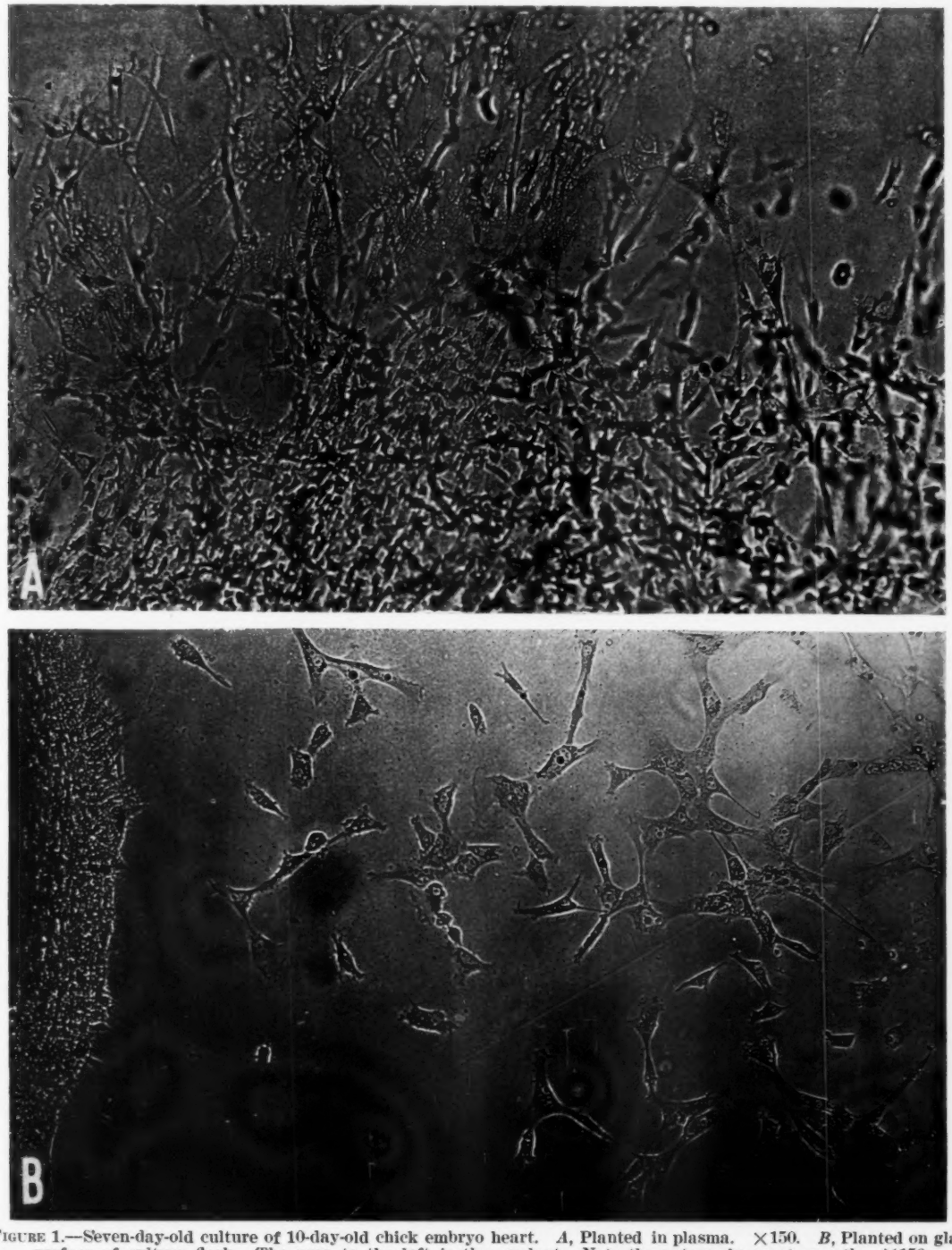

FIGURE 1.—Seven-day-old culture of 10-day-old chick embryo heart. *A*, Planted in plasma. ×150. *B*, Planted on glass surface of culture flask. The area to the left is the explant. Note the extremely scant growth. ×150.

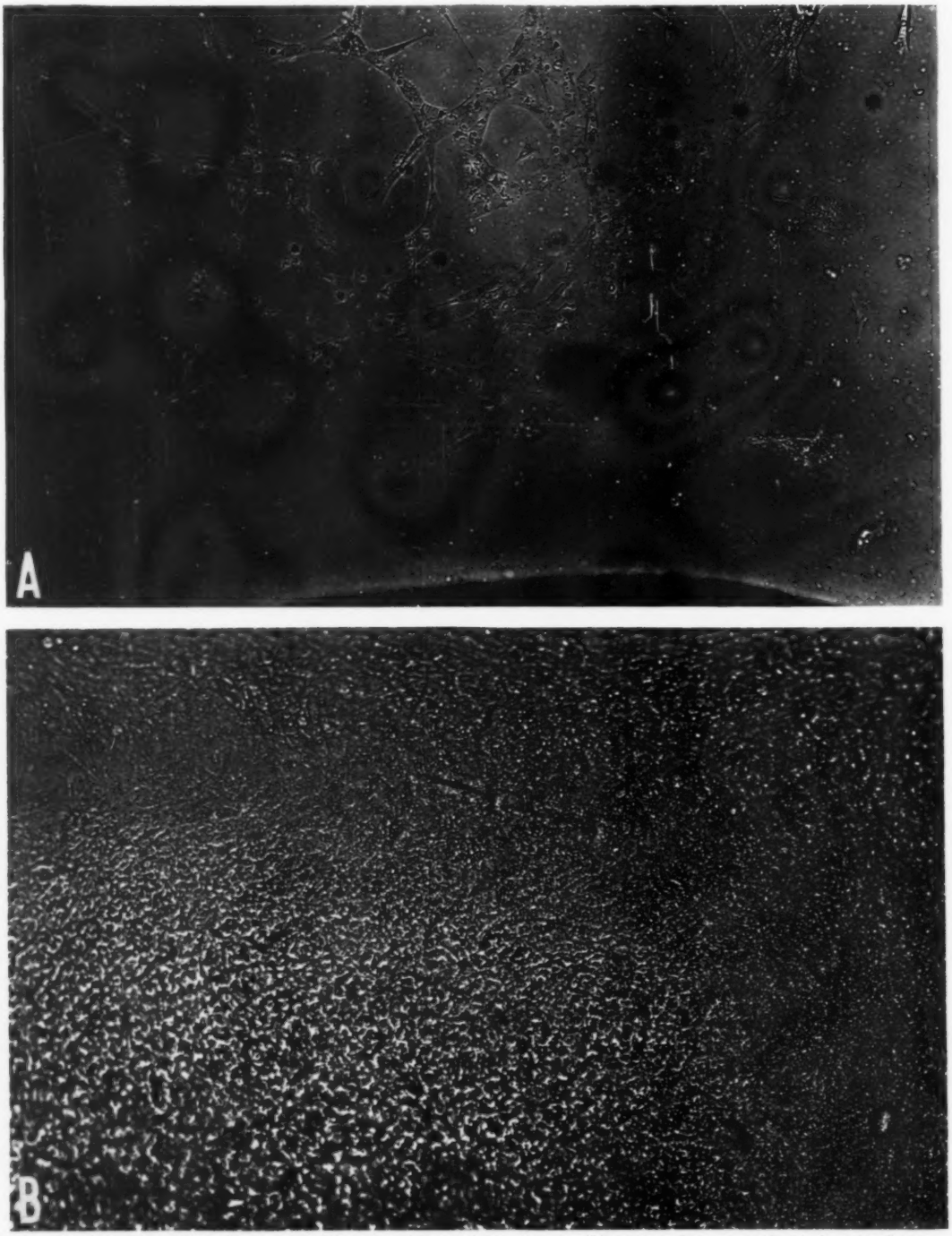

FIGURE 2.—Seven-day-old culture of 10-day-old chick-embryo heart. Planted under unperforated cellophane. *A*, The dark area near the bottom is the rounded-up explant. Note the scant growth. ×150. *B*, Growth so luxuriant, that cell detail was obscured by the cells being packed many layers thick. Compare with 2, *A*. ×150.

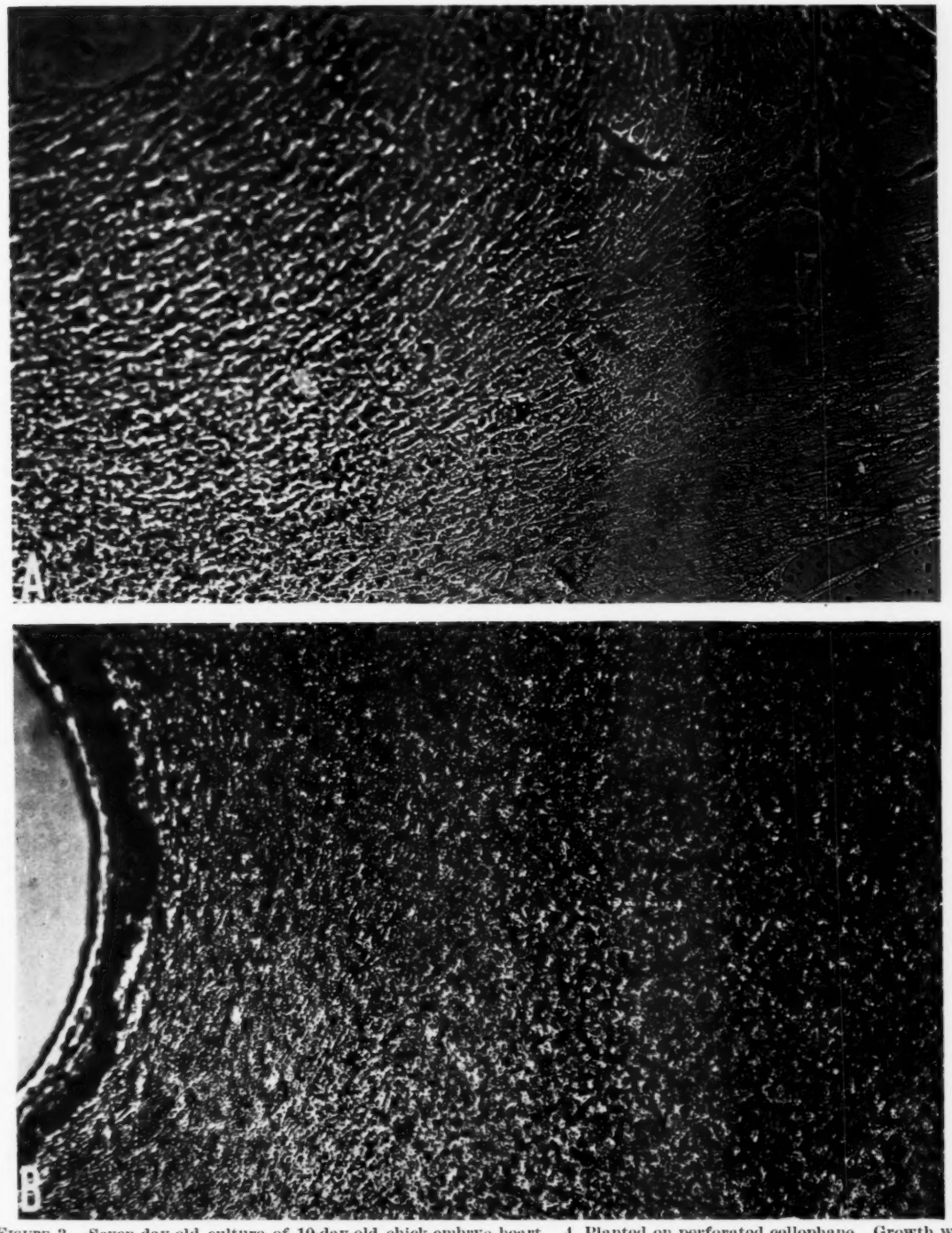

FIGURE 3.—Seven-day-old culture of 10-day-old chick-embryo heart. *A*, Planted on perforated cellophane. Growth was good but not luxuriant. ×150. *B*, Planted under perforated cellophane. The growth was luxuriant and extremely even. A part of one of the perforations is shown at the left. ×150.

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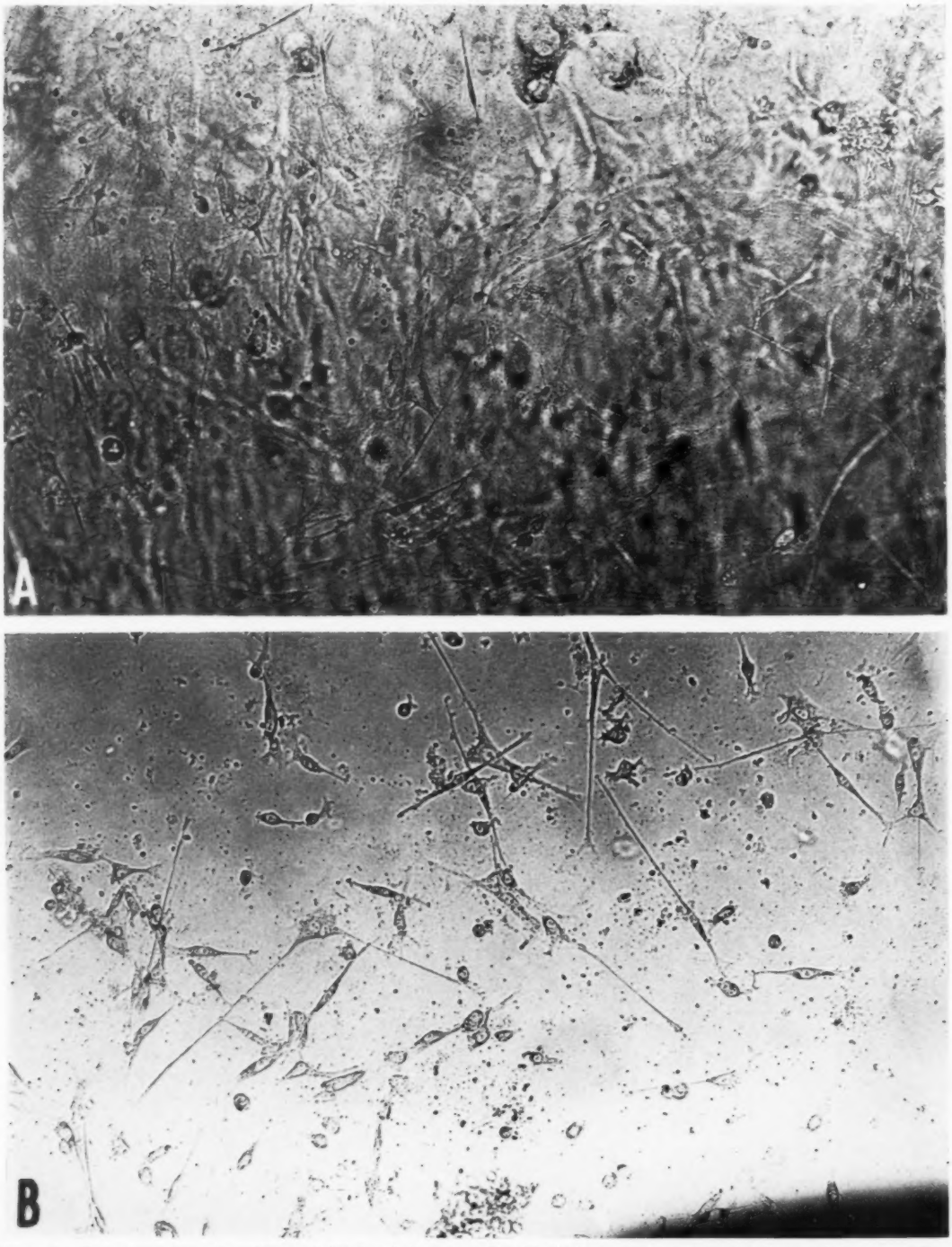

FIGURE 4.—Seventeen-day-old culture of fresh adult mouse fibroblasts from ventral abdominal musculature. *A*, Planted in plasma. Growth was only fair, while cell peripheries were very difficult to distinguish. ×150. *B*, Planted on glass surface of culture flask. The dark area shown at the bottom is the explant. The growth was extremely scant, and there was much cell rounding and necrosis. ×150.

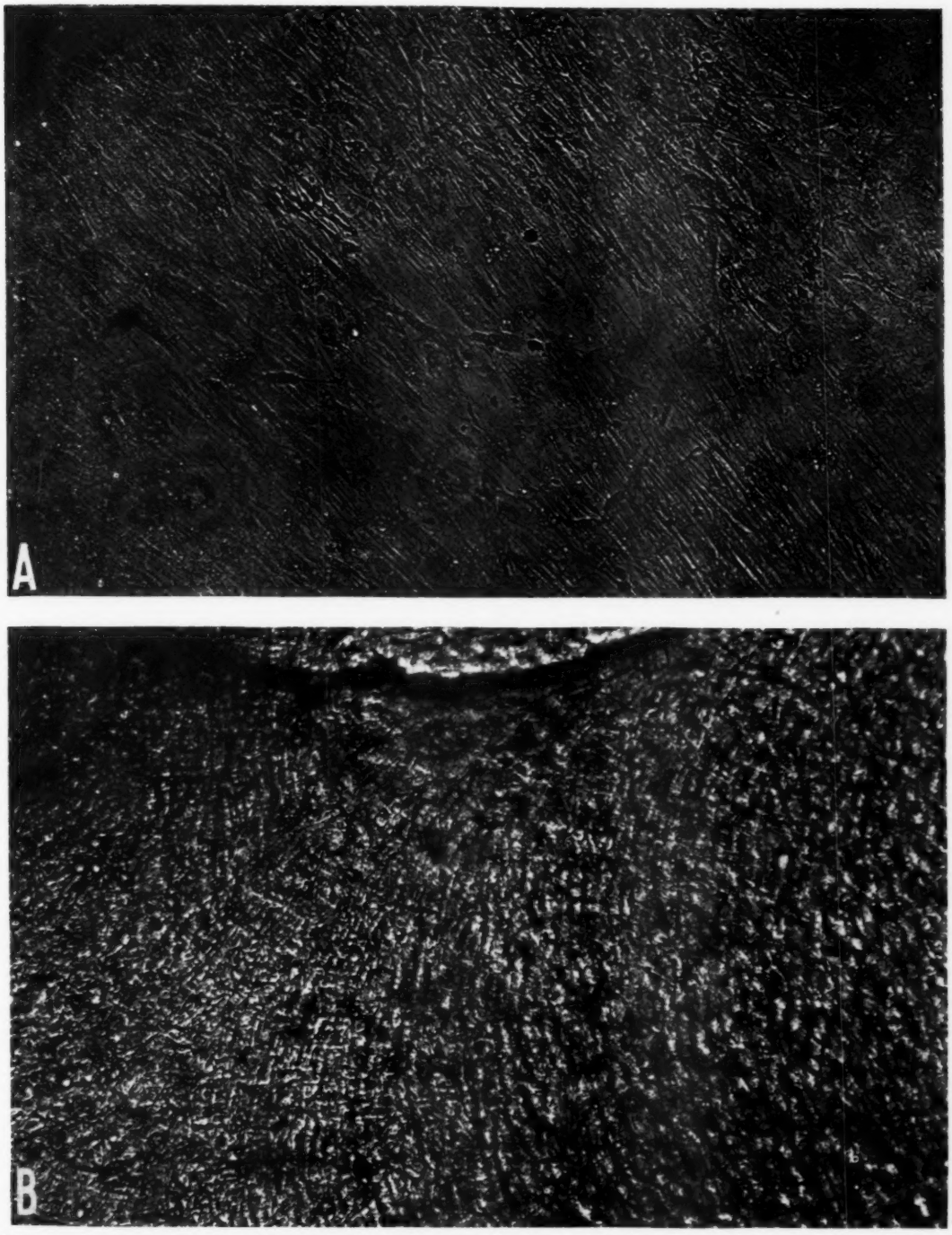

FIGURE 5.—Seventeen-day-old culture of fresh adult mouse fibroblasts taken from ventral abdominal musculature. *A*, Planted under unperforated cellophane. Growth was reasonably good, and better than in plasma. ×150. *B*, Planted on perforated cellophane. Growth was dense, and as frequently seen, here assumed a rather regular design relative to the perforations. ×150.

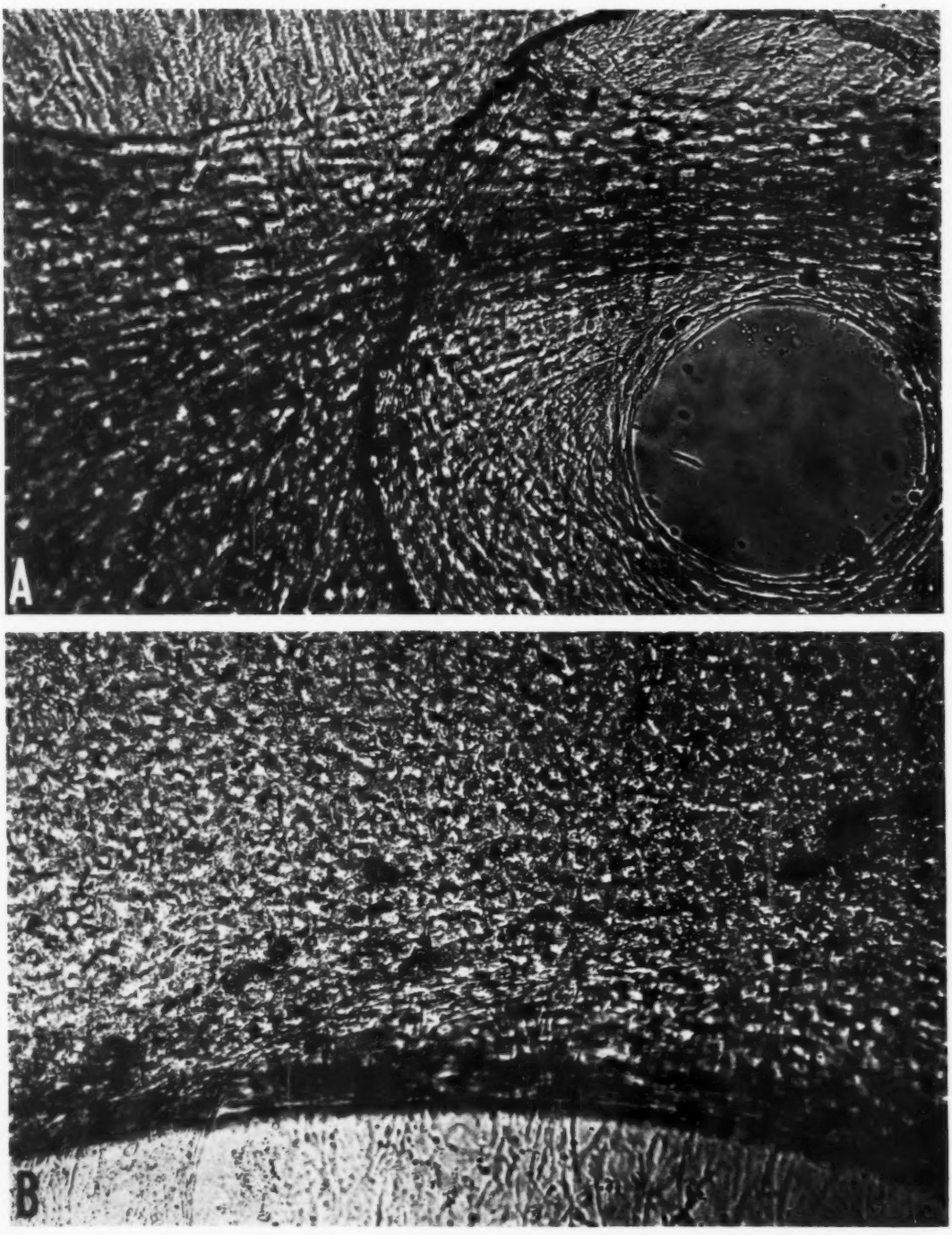

FIGURE 6.—Seventeen-day-old culture of fresh adult mouse fibroblasts taken from ventral abdominal musculature. *A*, Planted on perforated cellophane. One of the perforations is shown being penetrated by the cells, while cell layers on both upper and lower surfaces of the cellophane may be easily distinguished. ×150. *B*, Planted under perforated cellophane. Note the dense, even growth on the under surface of the cellophane. Near the bottom where the sheet of cells adherent to the cellophane has retracted, the very thin layer of cells adherent to the glass may be seen. ×150.

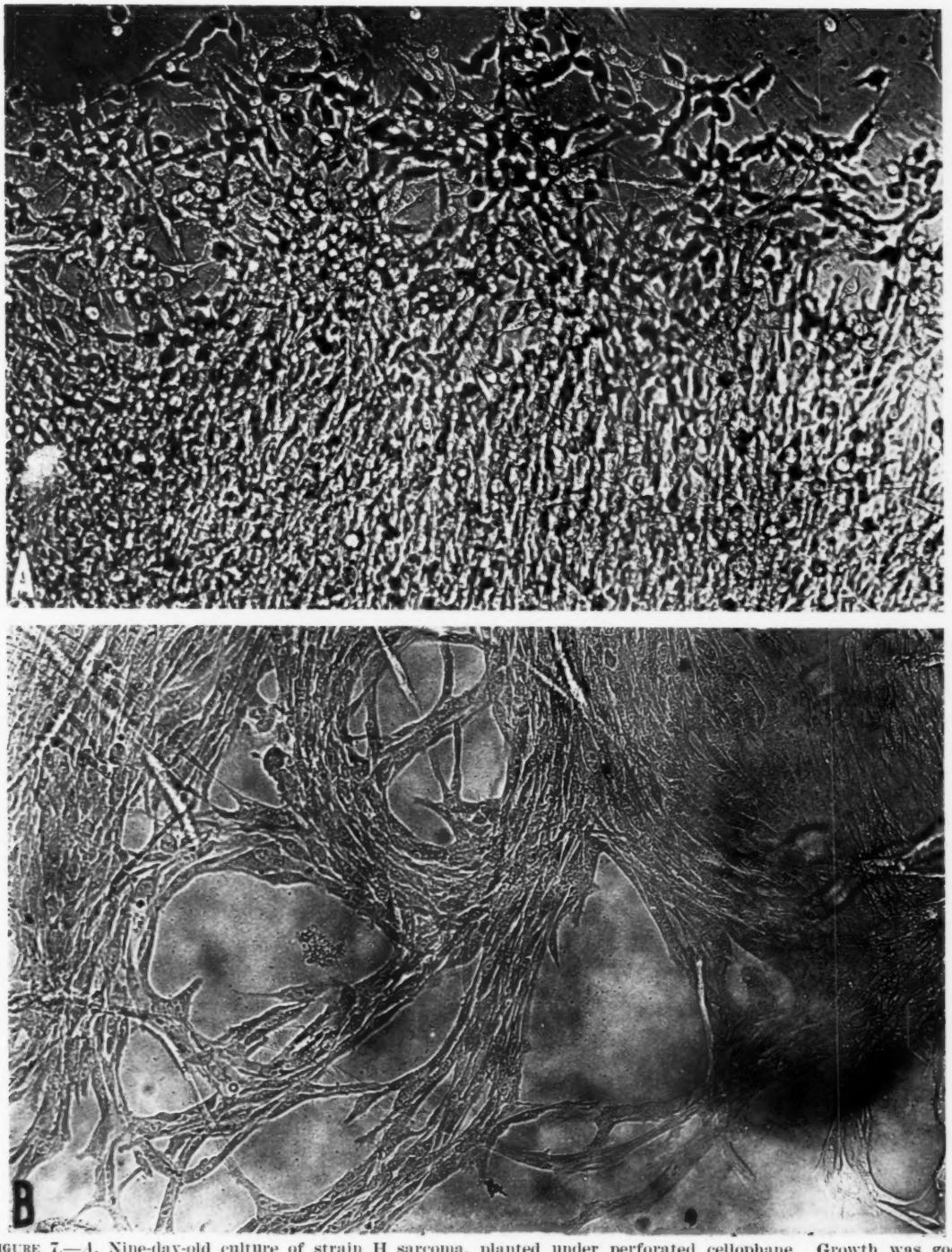

FIGURE 7.—*A*, Nine-day-old culture of strain H sarcoma, planted under perforated cellophane. Growth was good. ×150. *B*, Nineteen-day-old culture of a 3-year-old strain of normal adult mouse fibroblasts, planted in plasma. Growth was good. ×150.

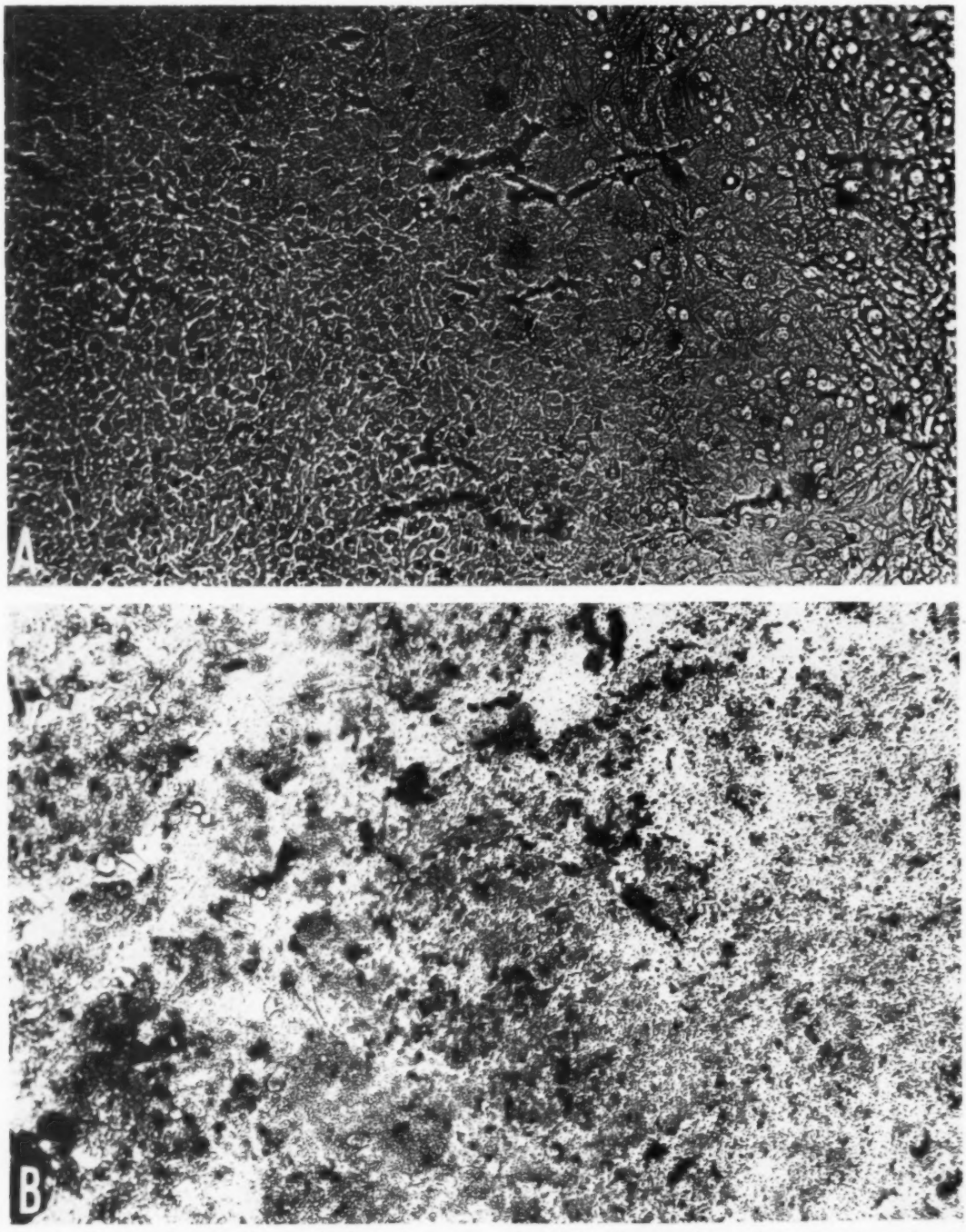

FIGURE 8.—Nineteen-day-old culture. *A*, Strain L sarcoma, planted in plasma and growing extremely well. ×150. *B*, A 3-year-old strain of normal mouse fibroblasts, planted under unperforated cellophane. This area was almost completely necrotic. ×150.

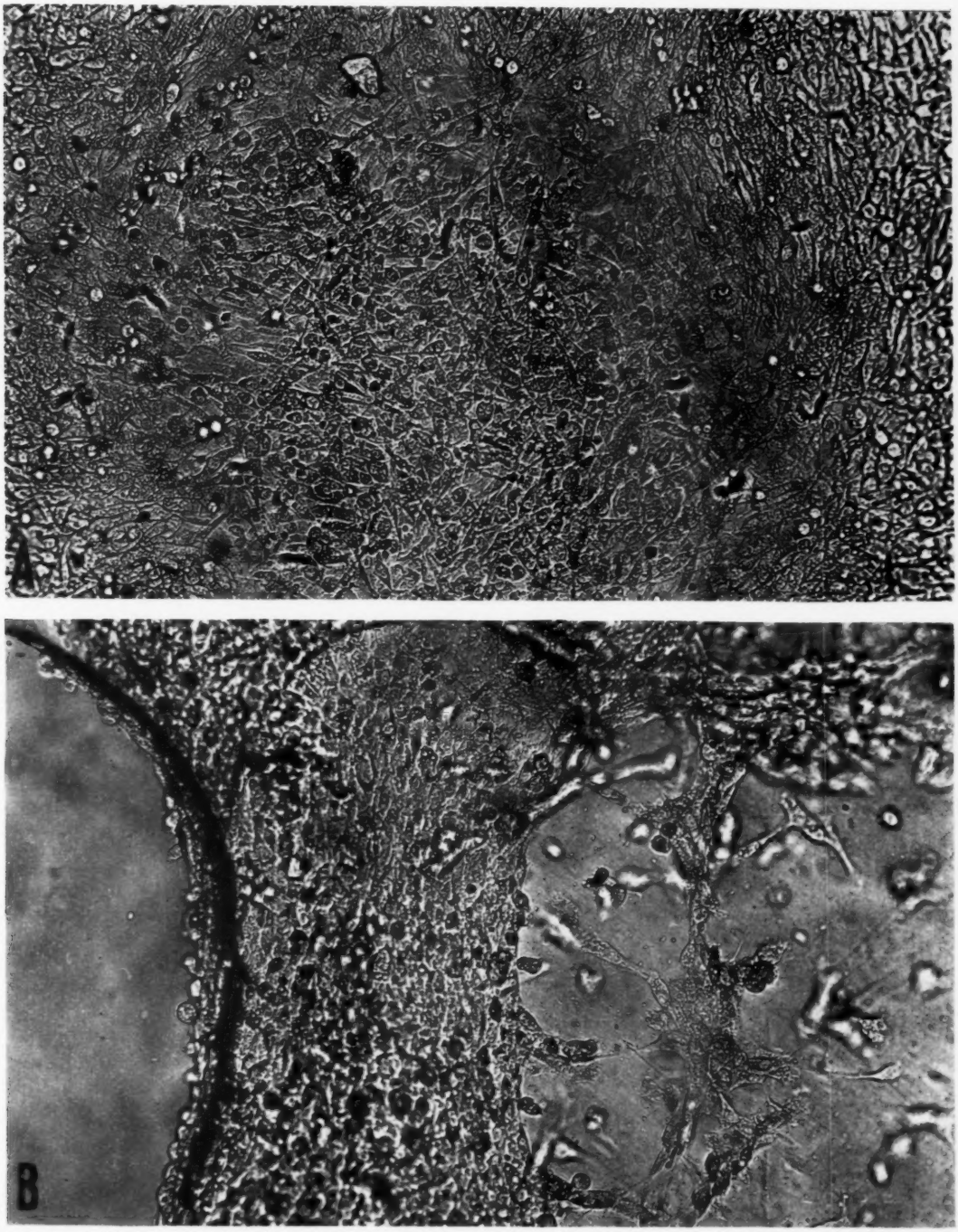

FIGURE 9.—*A*, Nineteen-day-old culture strain L sarcoma, planted under unperforated cellophane. The culture showed excellent growth. ×150. *B*, Three-year-old strain of normal mouse fibroblasts. Planted under perforated cellophane to show how the cell sheet adhered to the edge of the cellophane perforations, but pulled loose from the surface of the sheet. ×150.

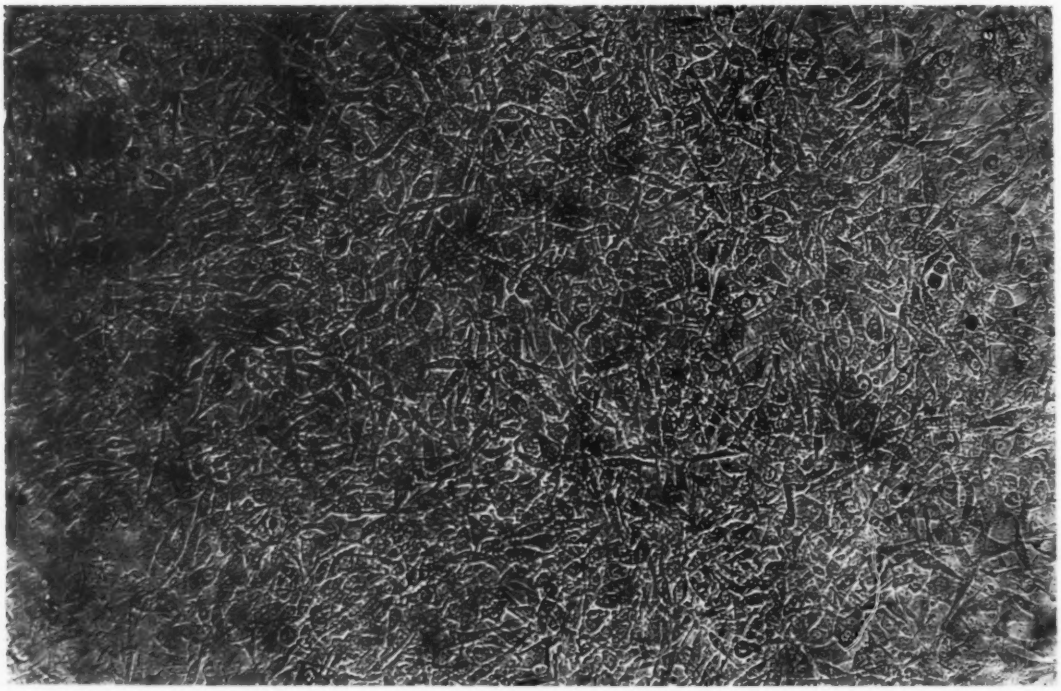

FIGURE 10.—Nineteen-day-old culture of strain L sarcoma grown under perforated cellophane. The cell sheet was evenly dense and luxuriant. ×150.

retracted, and were hanging in threadlike strands adherent to the edges of the perforations. Cultures of the strain L sarcoma (fig. 10) were luxuriant and reached the edge of the cellophane disks in both the 3.5- and 5.0-cm. flasks.

At 32 days, D 5.0 flask cultures of normal cells (fig. 11) showed scant growth and much necrosis. Cultures of the normal cells under perforated cellophane showed fair migration, but the cell sheets had pulled loose from the cellophane except at the edges of the holes, as shown in figure 12. Strain L sarcoma cultures under unperforated cellophane had a fairly even sheet of cells under the cellophane disk (fig. 13). The growth of the strain L sarcoma cells under perforated cellophane was more luxuriant and denser than any of the foregoing cultures, as may be seen in figure 14.

The sheet of cellophane with its adherent cells was pulled out from one of these flasks. Figure 15 is the hematoxylin-stained preparation [4] showing that few cells were left adhering to the glass floor of the flask.

DISCUSSION

From these studies, it appears that strains of sarcoma cells and fibroblasts can be grown luxuriantly in Carrel flasks under sheets of perforated cellophane. Growth under these conditions was superior to that seen on the glass surface of the culture flask alone or on the upper surface of unperforated cellophane sheets and appeared superior also to that on the upper surface of perforated cellophane. Even when growth was luxuriant in cultures planted on the upper surface of perforated cellophane, much of the growth resulted from the cell sheet's growing through the perforations and spreading on the lower surface of the disk.

With adult mouse fibroblasts, growth under perforated cellophane was good from both fresh and long-explanted tissue and covered the entire surface of the cellophane to the edge of the flask. The cell sheet was of irregular density apparently because of the difficulty the cells had in adhering to the cellophane, with the result that part of the cell sheets pulled away from it The most effective adhesion of the cell sheet appeared to be at the edge of the perforations. It is, therefore, possible that this growth can be improved by using closer spaced, finer perforations.

The growth of fresh chick heart and of two strains of sarcoma cells under perforated cellophane seemed to be of a luxuriance at least comparable with that seen in control plasma cultures and

[4] EARLE, W. R.: A procedure for the fixation, staining and mounting of whole mounts from tissue cultures grown in Carrel D 3.5 flasks. J. Nat. Cancer Inst. 8: 83–89 (1948).

possibly greater. With these cell types, the culture rapidly covered the area of the cellophane with a luxuriant sheet of cells. Growth was probably interrupted only because the mass of cells was such that it exhausted the volume of the culture medium within the interval between changes of medium.

Why cultures grow with greater luxuriance on the under surface of the perforated cellophane than on its upper surface is not clear. One factor may be that on the under surface the cells are more securely positioned and subjected to less danger of floating or being washed off. The possibility cannot be disregarded, however, that under the cellophane the cells thrive because of their ability to establish more favorable local culture conditions. For instance, they may establish a more favorable pH, a more favorable oxidation-reduction potential, or a more favorable zone of partial digestion of the culture media because of action of extracellular enzymes. The role of these various factors must await further study.

When a supernatant liquid culture medium such as embryo extract and horse serum is used, it has been uncertain whether the superior growth of cells in a plasma or fibrin matrix, as compared with growth of the cells on a glass surface, is due to the physical or mechanical role of the matrix, or whether the plasma or fibrinogen contributes some substances essential to or favoring the growth of the cells. There seems no reason to believe that

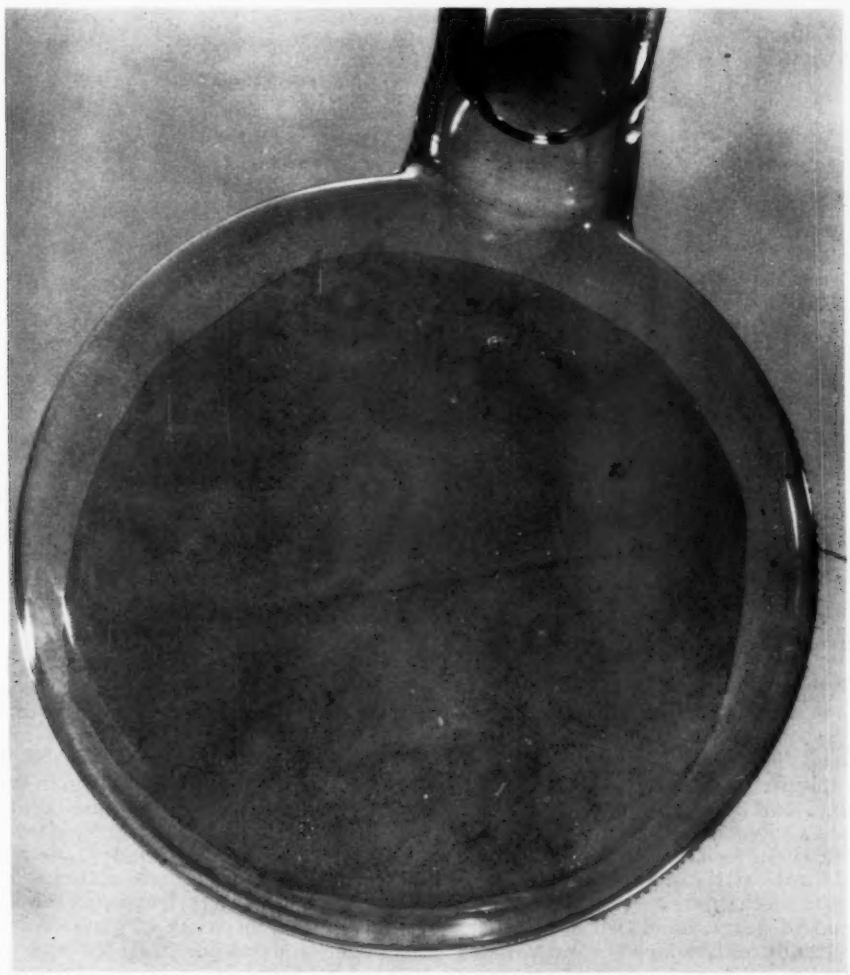

FIGURE 11.—Low-power photograph of hematoxylin-stained preparation of whole area of a 5.0-cm. flask culture, 32 days old, of a 3-year-old strain of normal adult mouse fibroblasts, described in series 7, grown under unperforated cellophane. The explant has washed entirely out of the culture. The cellophane does not stain.

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FIGURE 12.—Low-power photograph of hematoxylin-stained preparation of the whole area of a 5.0-cm. flask culture, 32 days old, of the same strain of normal mouse fibroblasts, grown under perforated cellophane. Note the characteristic design caused by areas of the cell sheet pulling loose from the cellophane but adhering to the edges of the perforations.

the cellophane contributes anything to the nutrition of the cells, and it appears that its role must be purely physical or mechanical. Since growth on and under the perforated cellophane has been at least as luxuriant as in the control cultures grown in plasma clots under the experimental conditions used, there seems no necessity for assuming that the role of the plasma clot is other than a physical or mechanical one.

Over a period of years, much work has been done with tissue cultures in which the cell clumps were situated on, and the cells were migrating on the surface of a glass cover slip or the surface of the glass of the culture vessel. Under the experimental conditions of the present study, cultures grown on the surface of the glass were not capable of producing luxuriant growth. In fact, as seen from data presented, if such glass-support cultures were used to test the horse serum-chick embryo extract medium, which gave luxuriant growth with cultures planted under perforated cellophane, one would conclude that this medium was poor or inadequate. Until the reason for such unsatisfactory behavior of glass support cultures is better understood, it is felt that conclusions should be most conservative concerning the nutrition of cells grown on such surfaces.

No accurate data are available on the weight

of the cells in these cultures. An estimate of aerobic oxygen consumption in a number of our standard D 3.5 plasma cultures by Dr. Dean Burk,[5] of the National Cancer Institute, indicated the presence of approximately 10 mg. of living tissue in each. The area and density of the 5.0-cm. flask cultures of sarcoma cells described herein seemed to indicate that the amount of cell material present is substantially greater than in those studied by Dr. Burk.

These preliminary investigations have not exhausted the potentialities of the method with respect to the size of the cultures that can be grown. An attempt is now being made to grow cultures in flasks 8 cm. in diameter. It is pertinent to think of the potentialities of the application of such massive plasma-free tissue cultures both in cancer research and in other fields.

No application of these findings with Carrel flask cultures has yet been made to other types of cultures, such as roller-tube cultures. It seems well worth while to explore the possibilities of using an internal sleeve of perforated cellophane in roller-tube cultures. In such preparations, the explant could be slipped down between the cellophane sleeve and the inner surface of the glass tube. The cellophane disks might also be tried with slide cultures to see if they can be used successfully as a substitute for plasma.

[5] Unpublished material.

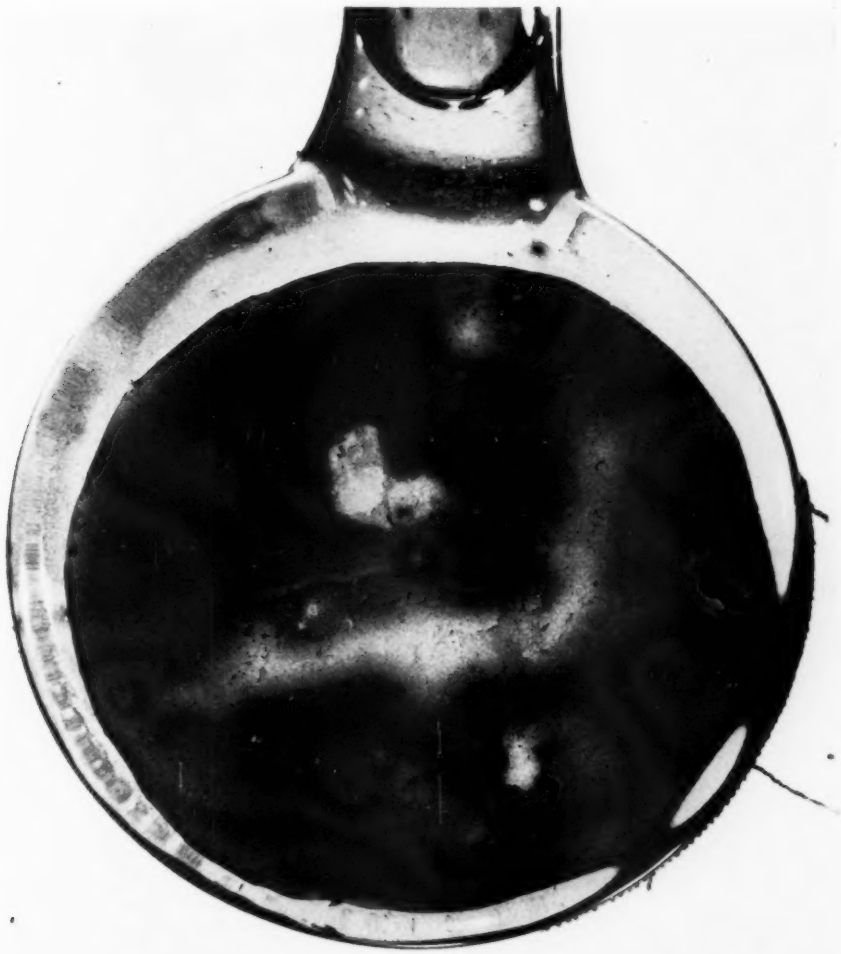

FIGURE 13.—Low-power photograph of hematoxylin-stained preparation of whole area of a 5.0-cm. flask culture, 32 days old of strain L sarcoma cells. Several small fragments have broken loose from the original explant and appear as irregular, black areas. The cell sheet was luxuriant, but of irregular density.

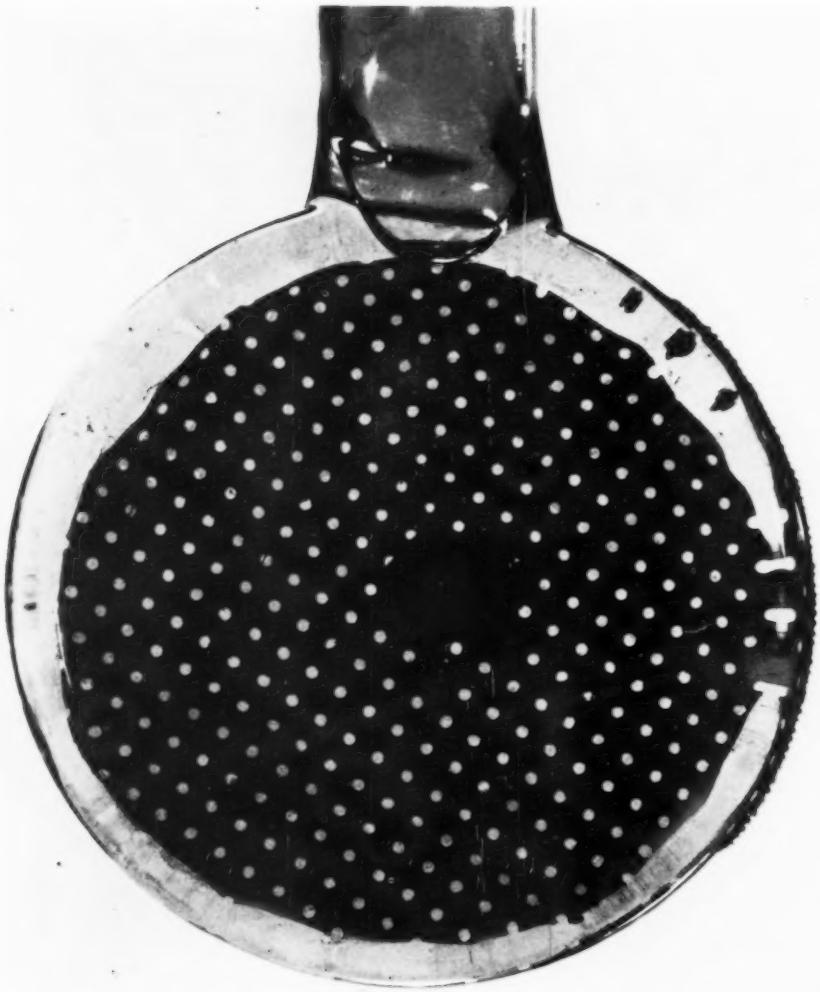

FIGURE 14.—Low-power photograph of hematoxylin-stained preparation of whole area of a 5.0-cm. flask culture, 32 days old, of strain L sarcoma cells. The culture was planted under perforated cellophane. Note the black area of the explant and the extremely dense growth of the culture.

SUMMARY

A comparison is made of the growth of freshly explanted chick-heart fibroblasts; freshly explanted, adult-mouse, subcutaneous fibroblasts; pure-strain, adult-mouse, subcutaneous fibroblasts; and Earle's strain H and L sarcoma cells; when grown in Carrel D flasks with various types of solid support for the cells, for from 12 to 96 days. All fluid culture media consisted of 40 percent horse serum, 20 percent embryo extract 1:1, and 40 percent saline. A study was made of growth of the cells in plasma; on the glass surface of the culture dish; on and under unperforated cellophane; and on and under cellophane perforated with one thirty-second inch holes on three thirty-seconds inch centers.

Growth of cultures on glass was so poor as to suggest they should not be relied on in studies of cell nutrition or the actions of the medium on the cell. The best growth was obtained in cultures planted under sheets of perforated cellophane.

This growth appeared to exceed that in the plasma clot and was so luxuriant that a thick mat of cells often reached to the edge of the 3.5- and 5.0-cm. culture flask in from 18 to 30 days. The ~~celophane~~ cellophane substrate is probably chemically inactive and apparently offers substantial advantages over the use of plasma. It should be particularly useful in studies of cell nutrition.

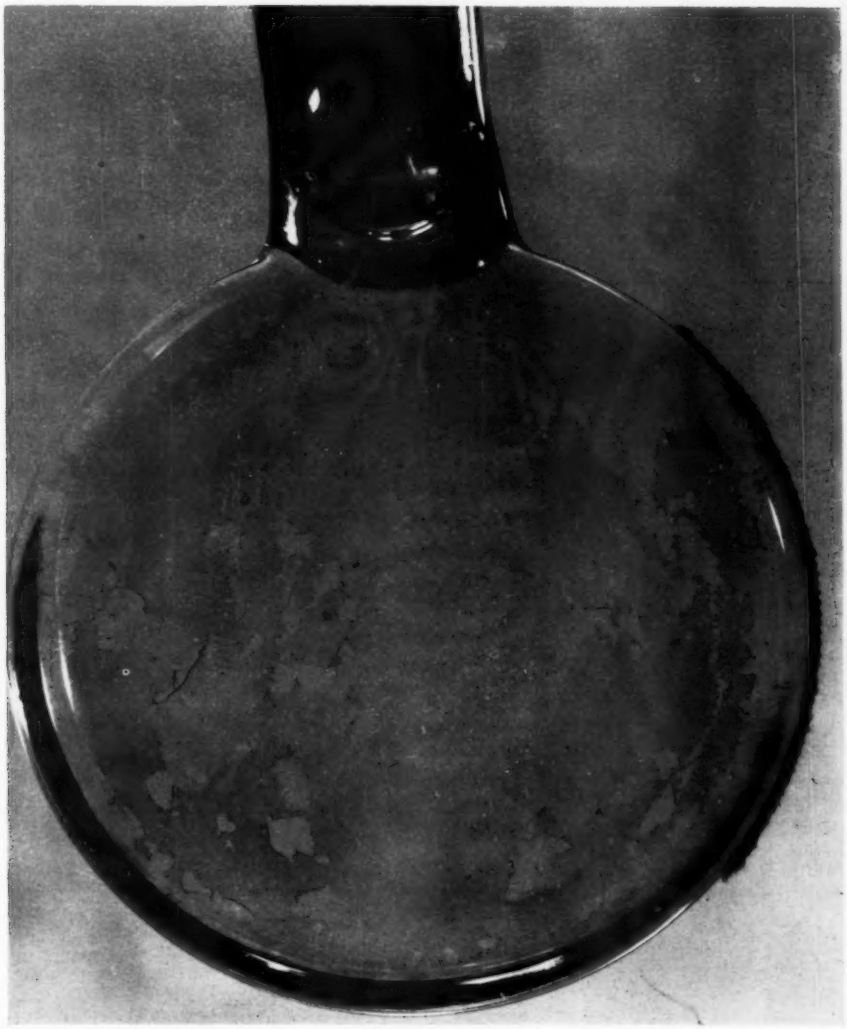

FIGURE 15.—Low-power photograph to show hematoxylin-stained cells adhering to the glass surface of a flask in a 32-day-old strain L sarcoma culture grown under perforated cellophane. The cellophane with its adherent cells was removed after fixation.

INHIBITION OF ANDROGEN-INDUCED COMB GROWTH IN THE CHICK WITH METHYLCHOLANTHRENE [1]

By ROY HERTZ, and WILLIAM TULLNER, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Recent studies of the mechanism of chemical carcinogenesis emphasized the initial growth-inhibiting effect of cancer-producing substances. Haddow et al.[2] discussed the factor of growth suppression in the early response to carcinogens and reviewed the literature pertaining to this phenomenon.

Studies were therefore undertaken on the qualitative and quantitative effect of various carcinogens upon several types of hormone-induced tissue growth. Observations on the effect of methylcholanthrene upon the comb-growth response to testosterone propionate [3] in the chick are reported herein.

MATERIALS AND METHODS

New Hampshire Red female chicks from the same flock were employed. They were maintained at 80° to 90° F. in electrically heated brooders and were fed a commercial starting mash ad libitum. Methylcholanthrene (melting point 177.0°—178.5° C.) was dissolved in ether at a concentration of 2 mg. per cubic centimeter; this solution was applied locally, by means of a fine pipette, over the comb surface at a daily dose of 0.05 cc. The combs of control chicks were painted with ether alone. Topical application of methylcholanthrene or ether was begun on the third day after hatching and continued to the time of autopsy. Starting on the seventeenth day of life, testosterone propionate was administered subcutaneously at a daily dose of 0.2 mg. in 0.2 cc. corn oil.

Daily observations were made of the gross size and shape of the comb, and at autopsy comb and body weights were recorded. All birds were autopsied 24 hours after the last treatment.

RESULTS AND DISCUSSION

The administration of androgen alone resulted in rapid and progressive growth of the comb. During the preliminary application of methylcholanthrene alone, no gross alteration in the character of the comb or surrounding tissue was apparent. However, after the first 5 days of combined androgen and methylcholanthrene administration, it was obvious that the comb-growth response was markedly inhibited in the methylcholanthrene-treated birds (table 1). After further androgen treatment up to 15 days, a marked reduction in the comb-growth response in the methylcholanthrene-treated birds continued to be quantitatively demonstrable. For example, the mean comb weight attained after 5 days of androgen administration in ether-treated chicks was 136.5 mg., whereas similar treatment with androgen in methylcholanthrene-treated chicks yielded a mean comb weight of only 39.5 mg. More strikingly, after 15 days of androgen treatment, the respective mean comb weights were 1,245.8 and 393.1 mg.

Qualitative observations on 1 series of 20 birds treated with androgen for 30 days indicated that the inhibitory effect became progressively reduced after about 20 days of treatment. This escape

[1] Received for publication November 21, 1947.

[2] HADDOW, A., SCOTT, C. M., and SCOTT, J. O.: Influence of certain carcinogenic and other hydrocarbons on growth rate of transplantable tumours. Proc. Roy. Soc. London, s. B, 122: 477–507 (1937).

HADDOW, A., and ROBINSON, A. M.: Association of carcinogenicity and growth-inhibiting in polycyclic hydrocarbons and other substances. Proc. Roy. Soc. London, s. B, 127: 277–287 (1939).

HADDOW, A.: Mode of action of chemical carcinogens. Brit. M. Bull. 4: 331 (1947).

[3] Testosterone propionate kindly furnished by Schering Corporation through the courtesy of Dr. E. R. Schwenk.

TABLE 1.—*Effect of administration of combined androgen and methylcholanthrene on comb-growth response*

Series	Number of chicks	Treatment with—			Age at autopsy	Body-weight mean and standard deviation	Comb-weight mean and standard deviation	Comb-body weight ratio mean and standard deviation
		Methylcholanthrene in ether	Ether	Androgen				
		Days	*Days*	*Days*	*Days*	*Milligrams*	*Milligrams*	*Milligrams*
A	5	0	0	0	22	151.4±24.9	35.6± 12.2	0.23 ±0.057
	7	19	0	5	22	129.0±22.3	39.5± 12.7	.31 ± .072
	8	0	19	5	22	126.6±42.5	136.5± 51.3	1.08 ± .357
	5	0	24	10	27	181.4±31.8	618.0±179.9	3.40 ± .904
	5	24	0	10	27	146.2±31.1	171.0± 27.1	1.19 ± .251
	6	0	29	15	32	257.1±22.7	1,245.8±290.2	4.80 ±2.972
	11	29	0	15	32	225.4±52.1	393.1±195.3	1.73 ± .783
	5	0	0	0	32	196.0±18.2	57.4± 19.0	.29 ± .043
B	6	0	0	0	27	161.6±30.9	60.8± 10.1	.38 ± .035
	6	0	24	10	27	216.3±30.7	578.3±132.9	2.75 ± .914
	11	24	0	10	27	182.6±13.6	236.3± 50.1	1.297± .280

from the inhibition was accompanied by the development of grossly abnormal combs in about 20 percent of the animals (figs. 1 and 2).

It is apparent that a decidedly abnormal growth pattern was induced. Such atypical tissue responses were not seen in the combs of birds treated with androgen alone for a comparable period of time. The ultimate fate of these combs and their histopathologic features are under study.

Some of the observations of Haddow et al.[4] indicated a general somatic inhibitory effect in addition to a local effect. A review of the respective body weights attained by control and experimental groups indicates no significant difference. This may be attributable to the relatively brief experimental periods involved. In addition, the highly specific and rapid local effect of the androgen may serve to dissociate the effect of the carcinogen upon the comb from its general somatic effects.

The growth-inhibitory effect of the carcinogen can occur in a tissue which is under maximal hormonal stimulation. Further studies are required to determine whether this effect is necessarily associated with the carcinogenic potency of such compounds as methylcholanthrene and other carcinogens. The availability of an external structure, which is quantitatively sensitive to hormonal stimulation for topical application of carcinogenic agents, permits further analysis of the interaction between the tissue-growth and carcinogenic processes.

[4] See first and last references in footnote 2.

SUMMARY

The comb-growth response to maximal doses of subcutaneously administered androgen in New Hampshire Red female chicks was substantially retarded by the topical application of methylcholanthrene. After approximately 20 days of combined treatment, this retardation became less marked; and in approximately 20 percent of the pullets, gross abnormalities in comb formation were observed. These effects were not associated with any general impairment of body growth. These observations support the thesis that tissue-growth suppression may play a very important role in the initial phases of the response to chemical carcinogens.

FIGURES 1 (left) AND 2 (right).—Grossly abnormal combs in chicks treated with methylcholanthrene for 39 days and androgen for 25 days.

INHIBITION OF ESTROGEN-INDUCED TISSUE GROWTH WITH PROGESTERONE[1]

By ROY HERTZ, C. D. LARSEN, and WILLIAM TULLNER, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

It has been known for some time that the characteristic progestational effect of progesterone upon the rabbit uterus can be eliminated by an excess of estrogen (*1–2*). Numerous studies have given approximations of the quantitative ratios involved in effecting this and other qualitatively antagonistic interactions between estrogens and progesterone (*3–6*).

The observations reported herein are concerned with the quantitative characterization of estrogen-progesterone antagonism and with some initial biochemical studies aimed at elucidating the mechanism of this antagonistic action.

EXPERIMENTAL PROCEDURE

Three-week-old New Hampshire Red chicks were maintained in electrically heated brooders and fed a commercial starting mash.[2] Stilbestrol was administered daily subcutaneously in 0.1 cc. corn oil, and progesterone was administered similarly in 0.2 cc. corn oil. Autopsies were performed 24 hours after the last of eight daily injections. The genital tracts were dissected rapidly and weighed to the nearest 5 mg. Animals of the same age and from the same flock were used throughout, and terminal body weights were recorded.

Serum calcium was determined by the method of Clark and Collip (*7*) and blood lipids by a modification of the method of Bloor (*8*). Blood was secured by cardiac puncture at autopsy.

RESULTS

The effect of increasing dosages of progesterone on the tissue-growth response to a maximally effective dose of stilbestrol is shown in table 1 and figure 1.

It is apparent that the degree of suppression of estrogen-induced tissue growth is proportional to the dose of simultaneously administered progesterone until a maximally effective dose of about 0.5 mg. daily is reached. With progressively increasing doses of progesterone up to 3 mg. daily, no further decrement in estrogen response is observed.

It is also noteworthy that in the presence of 0.5 mg. progesterone daily a sixteenfold increment in the dosage of stilbestrol is ineffective in reversing the progesterone-induced inhibition of tissue growth (table 1).

Limited observations have been carried out with estradiol with essentially the same results (table 1).

Progesterone administered alone has no apparent effect upon the chick genital tract (table 1).

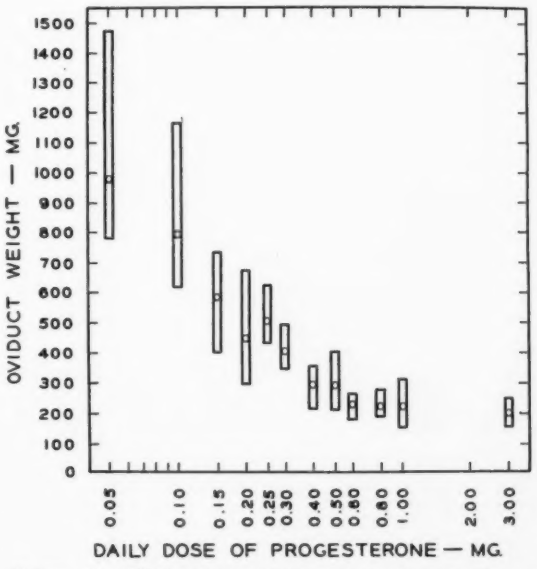

FIGURE 1.—Effect of increasing doses of progesterone tissue-growth response.

TABLE 1.—*Effect of increasing progesterone dosage on estrogen response*

Number of animals	Daily dose progesterone	Oviduct weight range	Mean response	Standard deviation
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*
44 [1]	0.00	590–1,270	930.45	166.54
7 [1]	.05	780–1,470	974.28	253.69
9 [1]	.10	620–1,165	792.2	192.03
9 [1]	.15	400–730	582.7	123.27
10 [1]	.20	295–670	440.26	111.20
10 [1]	.25	430–620	507.0	54.02
9 [1]	.30	340–490	400.5	44.40
17 [1]	.40	220–350	291.18	32.96
19 [1]	.50	175–400	281.84	57.40
9 [1]	.60	180–260	228.8	35.51
8 [1]	.80	190–270	220.0	31.62
9 [1]	1.00	150–310	220.0	43.01
10 [1]	3.00	150–250	198.0	29.81
10 [2]	.5	240–410	325.0	.....
9 [2]	.0	660–910	740.0	.....
8 [3]	.5	180–360	240.0	.....
10 [4]	1.0	13–24	17.5	.....

[1] Injected with 0.25 mg. stilbestrol in 0.1 cc. corn oil and indicated dosage of progesterone in 0.2 cc. corn oil daily for 8 days.
[2] Given 4 mg. stilbestrol plus indicated dose of progesterone daily for 8 days.
[3] Given 1 mg. of estradiol benzoate in 0.2 cc. corn oil plus indicated dose of progesterone daily for 8 days.
[4] Given indicated dose of progesterone and no estrogen; not significantly different from normal chick-oviduct weights given in tables 2 and 3.

[1] Received for publication November 21, 1947.
[2] Startena, Purina Mills.

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TABLE 2.—*Effect of progesterone on stilbestrol-induced lipemia*

Treatment with—	Oviduct weight	Blood lipids
	Milligrams	*Milligrams Percent*
Stilbestrol [1]	665	5,440
	785	5,800
	645	4,360
	630	5,400
	665	5,580
	650	5,440
	600	6,780
Mean	663	5,543
Stilbestrol [1] plus progesterone [2]	285	4,940
	235	4,100
	260	4,000
	260	4,480
	295	5,320
	255	4,680
	195	6,400
	230	3,240
	230	4,160
Mean	249	4,591
None	13	920
	21	1,140
	18	1,120
	16	1,040
	16	980
	17	1,020
	24	1,240
	13	1,100
	15	1,100
	22	1,080
Mean	17	1,074

[1] 0.25 mg. daily for 6 days.
[2] 0.5 mg. daily for 6 days.

TABLE 3.—*Effect of progesterone on stilbestrol-induced hypercalcemia*

Treatment with—	Oviduct weight	Calcium
	Milligrams	*Milligrams Percent*
Stilbestrol [1]	1,060	31.6
	1,040	28.0
	870	29.6
	1,010	25.0
	820	19.6
	1,090	32.0
	1,110	30.0
	950	26.9
	1,000	29.8
Mean	994	28.0
Stilbestrol [1] plus progesterone [2]	350	26.5
	380	20.4
	300	21.6
	330	22.0
	300	18.8
	270	24.4
	290	25.0
	300	21.0
	320	15.5
	240	14.8
Mean	308	21.0
None	18	10.0
	20	8.4
	20	11.0
	29	9.7
	21	10.0
	24	10.4
	21	9.8
Mean	22	9.9

[1] 0.25 mg. daily for 8 days.
[2] 0.5 mg. daily for 8 days.

Figure 2 shows the very substantial difference in size between the genital tracts of 3-week-old chicks, untreated, treated with stilbestrol alone, and treated with stilbestrol plus progesterone simultaneously.

The response to estrogen in the bird is characterized by a marked lipemia and calcemia (*9*). Accordingly, we undertook to determine whether the observed estrogen-progesterone antagonism might not entail some quantitative impairment in the mobilization of lipids and calcium in response to estrogen. Tables 2 and 3 show that a very marked lipemia and calcemia are obtained even in the presence of maximal suppression of estrogen-induced tissue growth by progesterone.

DISCUSSION

These data indicate that the growth response of the chick genital tract may be made up of two distinct components, namely, the basal amount of growth (up to about 250 mg.) which is resistant to progesterone inhibition, and the additional growth response (from 200 to 900 mg.) which is quantitatively sensitive to increasing doses of progesterone.

It is also of interest that once the system is saturated by a maximally effective dose of progesterone, a sixteenfold increase in the estrogen dosage will not reverse the inhibitory effect. This suggests that progesterone has a stronger affinity than estrogen for the reactive systems involved. The persistence of the marked lipemia and calcemia in the presence of a maximal degree of tissue-growth suppression by progesterone indicates that the serum lipids and calcium are not critically limiting factors in the oviduct response to estrogen. Moreover, it is apparent that progesterone does not prevent the absorption and at least the partial utilization of the estrogen.

The practical applicability of this remarkable inhibition of specific tissue growth remains to be evaluated. On the basis of the antifibromatogenic action of progesterone observed by Lipschütz and his associates (*10*) in the guinea pig, Goodman (*11*) employed progesterone in clinical cases of fibromyomas with reported success. However, Segaloff (*12*), employing radiological techniques of evaluation, observed no beneficial effects of progesterone in three cases of uterine fibroids.

The mechanism of steroid hormone antagonisms thus commands further study. An analogy may be drawn to the phenomenon of the inhibition of the biologic action of numerous vitamins and amino acids by their chemically related molecular analogs (*13, 14*). These antagonistic analogs are thought to compete with the related vitamin or amino acid for the same substrate in the reactive cell by virtue of their chemical simi-

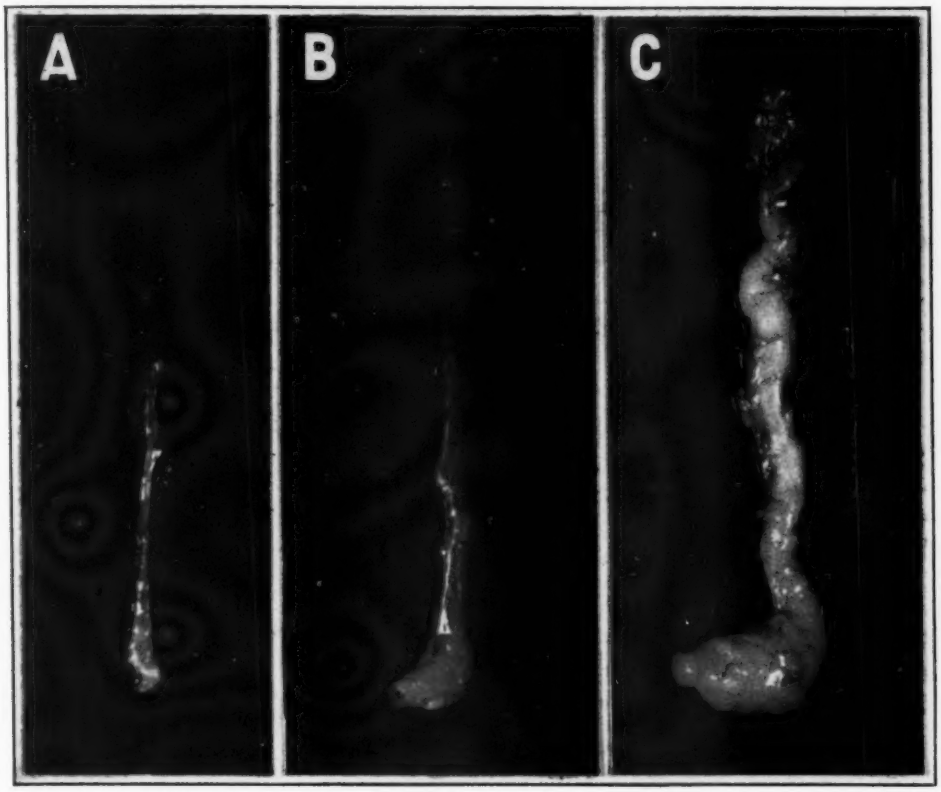

FIGURE 2.—*A*, Oviduct weighing 23 mg. from untreated chick; *B*, Oviduct weighing 23 mg. from chick given 0.5 mg. progesterone plus 0.25 mg. stilbestrol in oil subcutaneously daily for 8 days; *C*, Oviduct weighing 970 mg. from chick given 0.25 mg. stilbestrol in oil subcutaneously daily for 8 days.

larity to the parent substance. Many of the quantitative features of the sex-hormone antagonisms suggest that a similar mechanism may be operating. Thus the antagonistic action between stilbestrol and progesterone may be rationalized, particularly in view of Schueler's recent emphasis upon the significance of molecular dimension for estrogenic activity (*15*). These considerations are offered simply as a working hypothesis which may aid in the synthesis of additional hormone antagonists of therapeutic value.

SUMMARY

The simultaneous administration of progesterone with either stilbestrol or estradiol resulted in a marked inhibition of the estrogen-induced tissue growth in the genital tract of New Hampshire Red chicks. This inhibition was proportional to the dose of progesterone over a limited range. However, once a maximum degree of inhibition was obtained, further increments in progesterone dosage did not effect further decrements in estrogen-induced tissue growth. A sixteenfold increase in estrogen dosage did not reverse the inhibitory effect of a maximally effective quantity of progesterone. The lipemia and calcemia usually seen in estrogenized birds was unaltered by the simultaneous administration of progesterone in doses effecting a maximal degree of inhibition of estrogen-induced tissue growth in the genital tract.

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THIAMINE DEFICIENCY AND THIAMINE REQUIREMENTS IN C3H MICE [1] [2]

By Harold P. Morris and Celia S. Dubnik, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The role of specific vitamins in experimental cancer research can be studied precisely only after a careful study of the normal requirement of a particular animal species and strain (*1*, *2*, *3*). In attempts to measure the effect of thiamine on the growth of the already-established spontaneous mammary tumor in strain C3H mice, it was essential to study first thiamine deficiency and requirements in nontumor-bearing animals for periods at least equivalent to those used in the tumor studies (*2*, *3*). This procedure gave a fair appraisal of the effect of the vitamin in the animal without a tumor and corresponded to periods in which the influence of thiamine on the growth of the tumor could be successfully observed.

This report describes (1) acute thiamine deficiency in young and adult C3H mice, (2) thiamine requirements for growth, (3) the thiamine requirements for maintenance of body weight in adult mice, and (4) slowly induced or chronic thiamine deficiency.

A synthetic basal diet devoid of thiamine rapidly produces in both weanling and adult mice a deficiency syndrome, or acute thiamine deficiency. One of the early and well-recognized effects of acute thiamine deficiency in experimental animals is a loss of appetite. To evaluate the effect of thiamine deficiency and the concomitant decrease in food intake, the food intake of control animals was adjusted to that consumed by experimental animals.

After the response of an inbred strain of mice to a suitable diet devoid of thiamine was established, a study was made of the effects of adding graded amounts of thiamine to the basal thiamine-free diet. While Hauschildt (*4*) showed that growth in the albino mouse is in accordance with the amount of ingested thiamine, his experiments did not extend beyond 20 days after depletion of the initial thiamine reserves of the animals, nor were his observations made on mice regularly developing tumors.

It was believed that 3 weeks was too short a time to determine the influence of thiamine on the growth of the spontaneous mammary adenocarcinoma. If suboptimal amounts of thiamine would permit longer survival of the animals in a state of partial thiamine deficiency, then such a procedure might be used to measure the influence of thiamine on tumor growth. These experiments would also provide data on the quantitative requirements for thiamine of C3H strain mice for growth and maintenance of body weight and could be used also to determine whether or not the tumor-bearing animal required more thiamine than the nontumor-bearing animal.

These expectations have been realized. Certain levels of thiamine intake permitted relatively good growth of young mice for a time and delayed for considerable periods the usual symptoms occurring in thiamine deficiency. The regular production of this slowly induced, or chronic thiamine deficiency, its relation to the concomitant development of inanition, and its cure by thiamine supplementation are here described.

MATERIALS AND METHODS

Strain C3H mice from the National Cancer Institute stock were maintained in individual wire-cloth-bottom, metabolism cages in a room kept at approximately 78°–80° F. Tap water was available to the animals at all times. All diets were prepared at frequent intervals and stored in a cold room until used. Pyridoxine, riboflavin, calcium pantothenate, and nicotinic acid were thoroughly but lightly triturated in a mortar with a small portion of sugar before blending with the other dietary constituents in a power cake mixer.

The desired quantity of thiamine in the diet was secured by addition of proper amounts of sugar, to which a definite concentration of this vitamin had been added.

Table 1 gives the composition of the various diets.

Increased quantities of *dl*-α-tocopherol were added to diets 159 and 161 (table 1) to insure an excess of vitamin E under the conditions of restricted food intake during the terminal stages of the experiment. The amounts of the other vitamins were sufficient to meet the daily requirements at greatly reduced levels of food intake (*5*).

RESULTS

I. Acute Thiamine Deficiency in Young and Adult Mice

YOUNG MICE

Twelve litter-mate pairs of female strain C3H mice 25 days of age were used. One group was offered the basal thiamine-free diet 110 ad libitum while litter-mate controls were fed thiamine-supplemented diet 109 in amounts equivalent to those consumed by their respective pair mates. The thiamine-deficient groups made an average gain of approximately 2 gm. during the first week, which indicates considerable body stores of thiamine (fig. 1). Following this initial gain, the mice lost weight consistently and rapidly. The

[1] Received for publication August 5, 1947.
[2] Presented in part before the Division of Biological Chemistry, American Chemical Society, New York, September 1944.

TABLE 1.—*Composition of diets*

Constitutents	Diet															
	Control	Experimental														Control
	109	110, 110-1, 110-2	126	127	128	129	130	131	132	133	134	135	136	137	159	161
	Grams	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*
Vitamin-free casein (Labco)	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.	180.0	180.0	180.0	180.0
Dextrose (Cerelose)	554.5	554.5	563.0	563.0	563.0	563.0	563.0	563.0	563.0	563.0	564.0	563.0	563.0	563.0	563.0	563.0
Salt mixture (185a) [1]	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0
Crisco	200.0	200.0														
Wheat-germ oil	10.0	10.0														
Corn oil (fortified) [2 3]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0		
Corn oil															10.0	10.0
Coconut oil (containing A-ester+ *dl*-α-tocopherol) [4]															13.0	13.0
Lard			200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	187.0	187.0
Choline Cl	.5	.5	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
	Mg.	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*
Pyridoxine HCl	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Riboflavin	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Calcium pantothenate	100.0	100.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	40.0	40.0
Nicotinic acid	500.0	500.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
dl-α-Tocopherol [5]			100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	[6] 410.0	[6] 410.0
Inositol				10.0			10.0			10.0			10.0			
Thiamine HCl	40	0(110) 1(110-1) 2(110-2)	.1	.1	.8	1.0	1.0	1.5	2.0	2.0	3.0	5.0	5.0	10.0	1.0	10.0

[1] Morris and Robertson (*3*).
[2] Fortified corn oil contains vitamin A ester and calciferol equivalent in vitamins A and D to 2-percent dietary level of cod-liver oil.
[3] We are indebted to Distillation Production Co., Rochester, N. Y., for supplies of vitamin A ester, and to Mead-Johnson Co., Evansville, Ind., for supplies of calciferol.
[4] Vitamin A ester, 13 A units/1 gm. diet.
[5] The *dl*-α-tocopherol was dissolved in warm lard, before being added to the diet.
[6] The *dl*-α-tocopherol was dissolved in a small amount of coconut oil before being added to the diet.

thiamine-supplemented, restricted-food group did not show so much increase in body weight during the first week as did the deficient group. During the second and third weeks, the average weights of the two groups were essentially the same.

The average survival time of the deficient mice was 24.0±0.74 days, with a range of 20–29 days, while the average survival of the food-restricted, control animals was 22.5±1.00 day, with a range of 15–29 days. Three of the twelve control animals failed to survive as long as their deficient pair mates. During the fourth week, the deficient groups had almost ceased to ingest the deficient diet (fig. 1), and hence the control animals received on the average less than 0.2 gm. of food daily which contained 8 μg.[3] thiamine. This amount of thiamine is in excess of the daily requirement. Starvation, therefore, must have been the cause of death of these control animals.

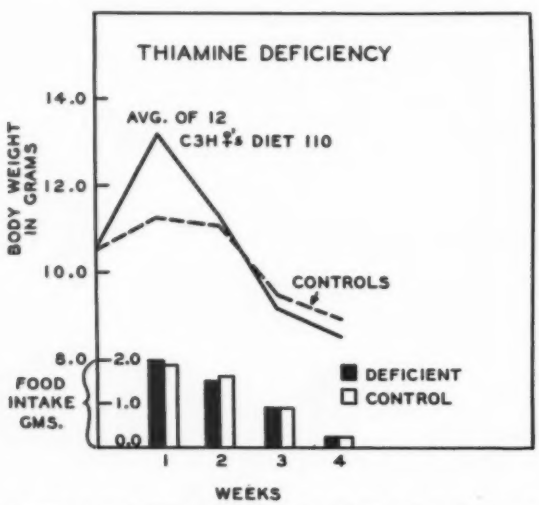

FIGURE 1.—Average body weight of 12 pairs of young female C3H mice fed the same quantity of food. Controls, broken line, given thiamine; and solid line, without thiamine.

ADULT MICE

Female strain C3H mice 3 months of age were placed on the thiamine-deficient diet 110. These animals maintained their initial body weight for the first week. They lost weight rapidly during the next 2 weeks (fig. 2, curve A). Control animals restricted to the same amount of food as consumed by the deficient animals began losing weight immediately, but after the first week at a somewhat less rapid rate than the deficient animals (fig. 2, curve B). After 3 weeks, the body weights of both groups were the same.

The average survival of the adult deficient animals was 24.0±0.7 days, with a range of 19–28 days. All the control animals survived as long as their deficient pair mates. This survival is in

[3] The abbreviation μg. is used herein instead of the word "microgram."

distinct contrast with that of the young animals where 25 percent of the control, food-restricted animals succumbed before their deficient pair mates.

It seems probable that the survival of all the control adult animals until their deficient pair mates died was related to the slightly higher food intake of the deficient adult animals, compared with the deficient weanling animals (food intake, figs. 1 and 2). This was especially evident near the terminal part of the experiment.

SYMPTOMS OF ACUTE THIAMINE DEFICIENCY

In this rapidly produced or acute thiamine deficiency state, symptoms were observed before death in both young and adult animals, but much more frequently in the younger group.

These symptoms usually appeared between the twentieth and the twenty-fifth day in both groups but particularly in younger animals sometimes developed a few days earlier. First noted was a condition of hyperirritability and tensing of the muscles on stimulation, especially. Two to three days later if animals were suspended by their tails, they showed a tendency to spin. When dropped to a plane surface, the animals showed a variety of characteristic movements depending on the severity of symptoms. Some appeared exhausted and could be made to move only after prolonged prodding. Others became rigid after being released, their bodies assuming the position of an inverted arch with legs thrust stiffly outward. The animals would roll over from side to side several times before they relaxed. At other times, animals after being picked up and then released would attempt to walk away. They would be unable to move either forward or backward but instead would pivot. The animals appeared to be able to move their forelegs easily and rapidly at this stage, whereas they moved their hind legs slowly and with great difficulty. The hind legs were not considered to have a true paralysis since the symptoms were manifested only after stimulation. When the animals were observed in their cages 1 or 2 days before death, they moved only with great difficulty and slowly. They usually felt cold to the touch, and their breathing was rapid and shallow.

Considerable differences were noted in the severity of the symptoms in different animals, but seldom did an animal survive more than a few days after developing severe symptoms. The clinical manifestations of thiamine deficiency in the mouse resembled but were not identical with symptoms described for chronic thiamine deficiency in the rat by Prickett, Salmon, and Schrader (*6*).

II. Thiamine Requirement for Growth

Twelve groups, each comprised of 10 weanling strain C3H mice reared on stock diet 77 (*5*), were used in the first experiment. These animals were weaned between the twenty-fifth and the twenty-seventh day of age. Diets 126–137, inclusive, containing varying amounts of thiamine were used (table 1).

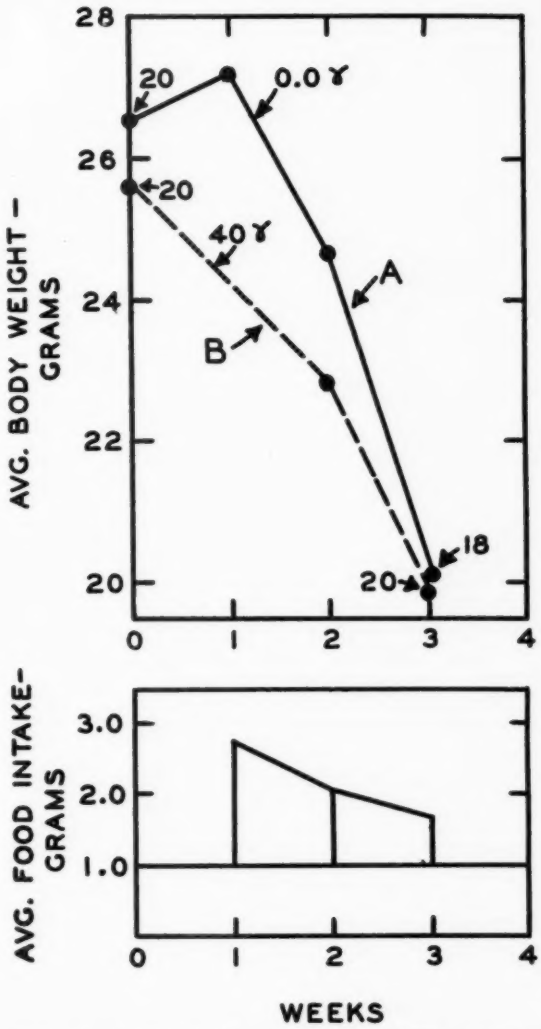

FIGURE 2.—Average body weight of adult C3H nontumor-bearing mice fed the same quantity of food with and without thiamine. Curve A, mice receiving no thiamine; curve B, mice receiving 40 μg. thiamine per gram of food.

All groups gained weight for 10 days. The animals on diets 126 and 127 (fig. 3) began losing weight after the tenth day. This loss was very rapid, and death occurred in from 1 to 2 weeks.

The animals on diets 128, 129, and 130, containing 0.8 and 1.0 μg. thiamine per gram, continued

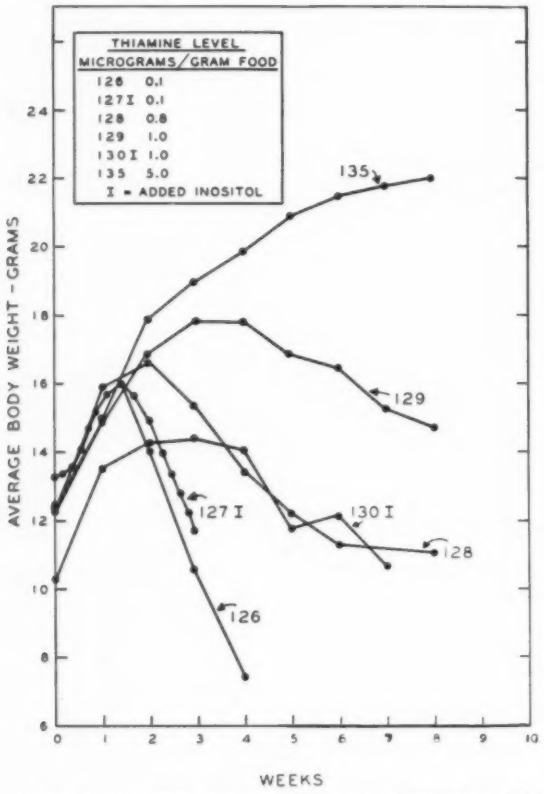

FIGURE 3.—Average gain in body weight of weanling C3H mice fed graded amounts of thiamine and thiamine+ inositol over an 8-week period.

to gain in weight for 2 more weeks but at a slower rate (fig. 3), after which these animals also began losing weight. The animals lost weight for the following 4 or more weeks, at which time they began developing clinical symptoms of thiamine deficiency. These animals usually died within a very few days after the first appearance of deficiency symptoms. The average length of survival on diet 128 was 44 days, although 3 mice lived 60–63 days.

Animals receiving 1.5 μg. (diet 131) to 10 μg. (diet 137) thiamine per gram of food (table 1) did not develop any manifestations of thiamine deficiency during the 8-week period of observation. The average increase in body weight at the 1.5 μg. level of thiamine was somewhat less than at the 2-μg. level or more. This is evident in figure 4. Animals on the 1.5-μg. level ingested on the average 3.6 μg. to 4.6 μg. thiamine, while at the 2-μg. level they ingested 5–6 μg. (fig. 5, B). Growth appeared to be much the same when dietary levels of 2 to 10 μg. thiamine per gram of food were used, indicating that when the basal diet contained 2 μg. or more of thiamine per gram, full growth stimulation was obtained.

Food intake for each week of the 8-week period was studied. Part of the data is illustrated in figure 5. The food intake remained practically constant for groups on diets containing 1.5 μg. or more of thiamine per gram (fig. 5, A). All groups made full growth on that amount of thiamine, except the group receiving the smallest amount of thiamine, in which growth was somewhat less than for the groups on higher levels of thiamine. Three groups receiving thiamine levels lower than 1.5 μg. per gram maintained approximately the same food intake for from 1 to 4 weeks after which the intake dropped below that in animals making full growth (fig. 5, A, groups 126, 128, and 129). On a weekly basis, the relative difference in the average daily thiamine intake in these three groups (fig. 5, B) was greater than that noted for their food intake (fig. 5, A). This difference was caused by the feeding of different levels of thiamine.

The average minimum thiamine intake, where no deficiency effect was noted on either growth or clinical symptoms, was between 5 and 6 μg. per mouse per day (fig. 5, B, curve 132). The thiamine intake was constant in this group throughout the 8-week period and under our experimental conditions appears to be the minimum daily requirement for optimum growth of young C3H mice. A considerably higher average daily thiamine in-

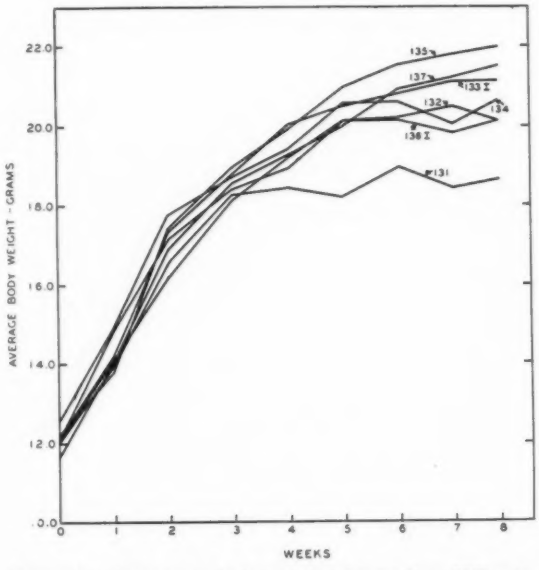

FIGURE 4.—Average gain in weight of weanling C3H mice fed sufficient amounts of thiamine to prevent development of clinical symptoms of thiamine deficiency. The numbers on the curves refer to the diet number used for each group.

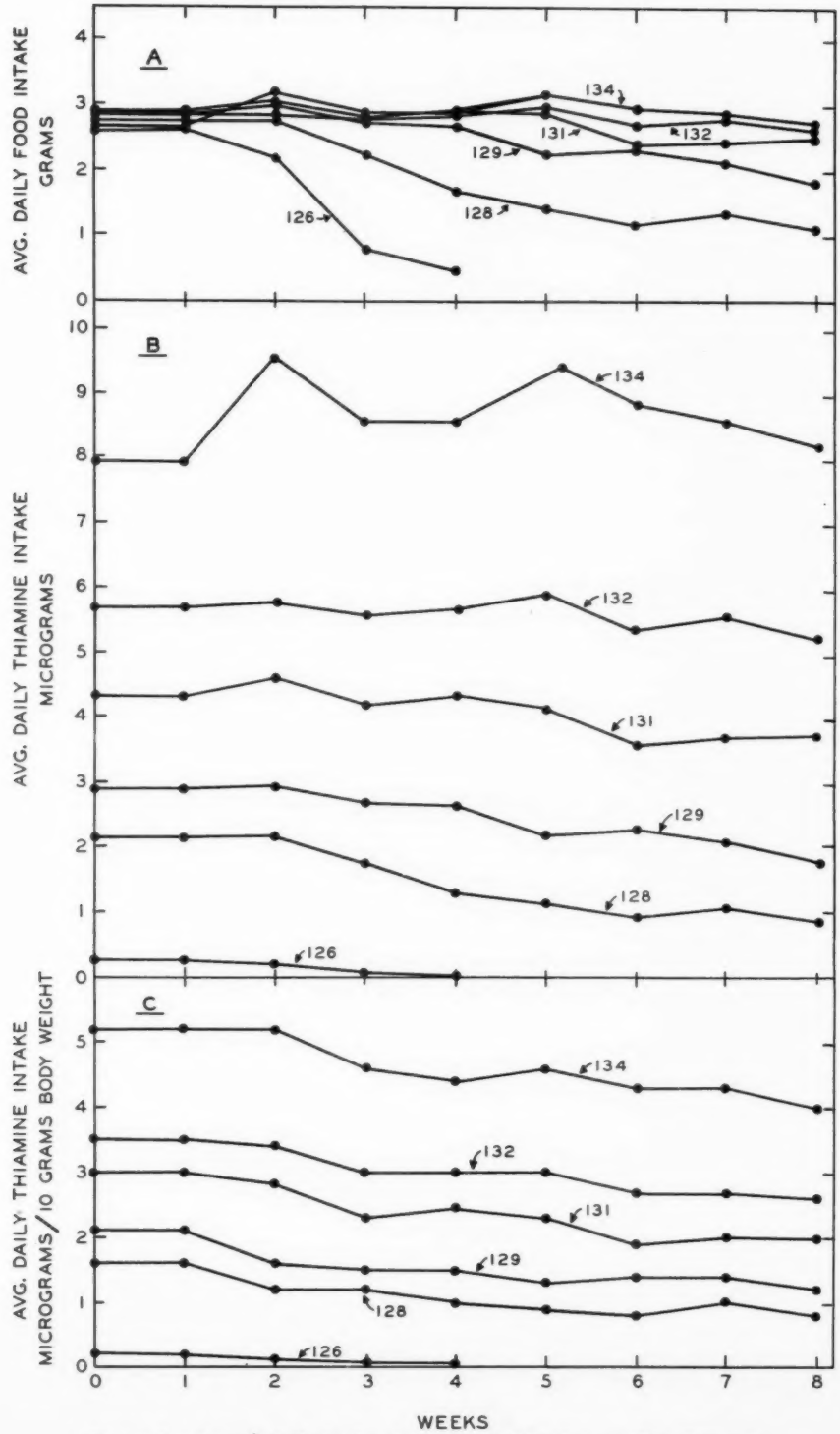

FIGURE 5.—Average daily food and thiamine intake for weanling C3H mice.

take (fig. 5, B, curve 134) did not result in any increased rate of growth.

Since growth expressed as increase in body weight was used as a criterion for estimating the thiamine requirement, the question arose what was the relation of thiamine intake when expressed per unit of body weight. It was found that during the first 3 weeks in five of six groups the thiamine intake decreased (fig. 5, C). After the third week, even in mice suffering from thiamine deficiency (fig. 5, C, curves 128 and 129), the thiamine intake remained practically constant at 1 μg. daily per 10 gm. of body weight for group 128 and 1.5 μg. daily for group 129, despite decreasing food and thiamine intake. In the presence of suboptimal intake of thiamine, after the mice began losing weight, such loss occurred simultaneously with decreased food intake just as was observed in acute thiamine deficiency.

Per unit of body weight, the difference between an adequate and an inadequate daily thiamine intake is less than is the actual difference. Thus the average of 2 μg. daily per 10 gm. body weight during the sixth to eighth weeks for group 131 just slightly below an adequate level was only 0.5 μg. per day per 10 gm. body weight less than for group 132, which ingested an adequate amount of thiamine. The actual difference, however, in average daily thiamine intake for these two groups was from slightly more than 4 μg. to 5.5, respectively.

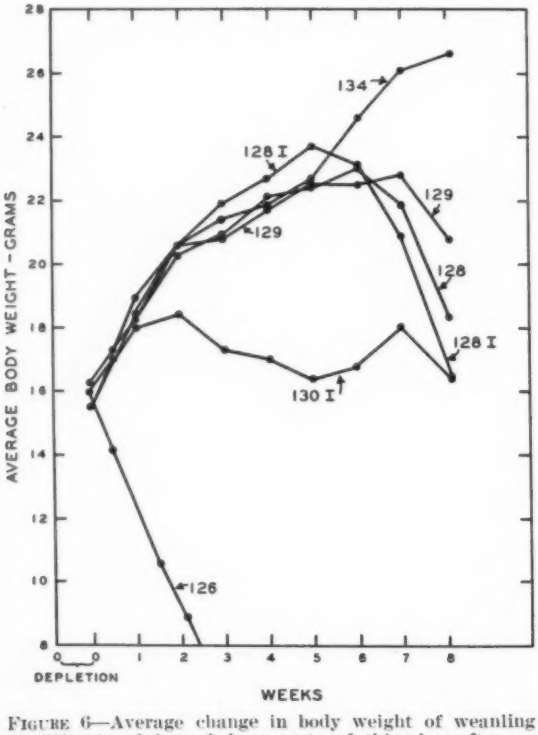

FIGURE 6—Average change in body weight of weanling C3H mice fed graded amounts of thiamine after an initial thiamine-depletion period.

GROWTH FOLLOWING DEPLETION OF INITIAL THIAMINE RESERVES

The weanling C3H mice from stock diet 77 (*5*) were given diet 126 until their thiamine reserves had been depleted as evidenced by stationary weight. This depletion required from 10 to 16 days, after which the animals were placed on the basal diet supplemented with graded quantities of thiamine (diets 126, 128, 128I,[4] 129, 130I, and 134), and the changes in body weight were observed for the following 8 weeks. All the animals except group 126 gained weight for 2 or more weeks after the depletion period. The group on diet 126 (low thiamine) began losing weight immediately and rapidly, surviving only 15 days on the average (fig. 6). Only 1 of 11 animals maintained on diet 126 showed mild clinical evidences of thiamine deficiency, which were characterized by labored breathing and difficulty in maintaining balance. When at rest, the animal took a humped position with head resting on the floor of the cage, and it seemed to have difficulty in moving or bending its hind legs. The symptoms were not strikingly different from those previously described but were more nearly like the symptoms occurring near the terminal stages of the deficiency. The other 10 animals died without showing gross clinical evidences of thiamine deficiency. Chronic thiamine deficiency was produced in about the same length of time when the animals were first depleted of their initial reserves as when they were taken off stock diet at weaning time and placed on diets containing an insufficient amount of thiamine.

INOSITOL

A preliminary observation suggested that arrested growth on a suboptimal level of thiamine (1 μg. per gram of food) may have been partially alleviated by the addition of inositol to the diet. Several groups of mice were fed diets containing inositol with graded amounts of thiamine. Diets 127I, 130I, 133, and 136, without depletion, and diets 128I and 130I, with initial depletion, were used (fig. 3 and fig. 6). Inositol added to diets containing inadequate, suboptimal, or excessive amounts of thiamine did not enhance the growth of mice. In one group diet 130I (fig. 6, curve 130I), growth was much depressed after the first week following depletion.

[4] I following a diet number indicates that diet contained added inositol.

III. Thiamine Requirement for Maintenance of Body Weight in Adult Mice

Fifteen female C3H mice 5 months of age were taken off the stock diet and placed on basal thiamine-deficient diet 110 to which 2 μg. thiamine had been added per gram of diet. There was a slight increase in body weight during the first seven weeks (fig. 7). A reduction in the amount of thiamine after the eleventh week to 1 μg. per gram of diet (diet 110–1, table 1) caused a loss in body weight during the next 5 weeks (fig. 7), at which time spontaneous mammary tumors began to appear. Mice developing tumors were removed from the experiment, so that at 20 weeks only eight animals were left. Body weight decreased less between the sixteenth and twentieth week than between the eleventh and sixteenth week. The daily food intake decreased from 2.7 to 2.1 gm. per day during the eleventh to twentieth week (fig. 8, A).

On the diet containing 2 μg. thiamine per gram, the average daily intake of vitamin ranged from 5.0 to 6.4 μg. and averaged 2 μg. per 10 gm. of body weight. On the 1μg. per gram diet, the average daily intake ranged from 2.1 to 2.5 μg. (fig. 8, B) and averaged 1 μg. of thiamine per 10 gm. body weight daily (fig. 8, C). The thiamine intake per unit of body weight appears to have remained exceedingly constant throughout the period of observation. No symptoms of thiamine deficiency were observed during the 11-week period when the mice received 2 μg. thiamine per gram of food. Between the eighteenth and twenty-fifth weeks of the experiment, or after 7 to 14 weeks at the 1-μg. level of thiamine intake, four of nine mice developed mild symptoms of deficiency characterized by dragging of the hind legs. A diet containing 1 μg. of thiamine per gram was therefore deficient in this vitamin. The minimum thiamine requirements of adult C3H mice thus appears to be somewhat higher than 2.5 μg. but probably less than 5 μg. to 6 μg. per day.

IV. Slowly Induced or Chronic Thiamine Deficiency

The slowly induced, or chronic-thiamine-deficiency syndrome, as produced previously by feeding a diet containing a small but insufficient quantity of thiamine, was further investigated. Although the symptoms appeared the same as those observed in animals ingesting diets devoid

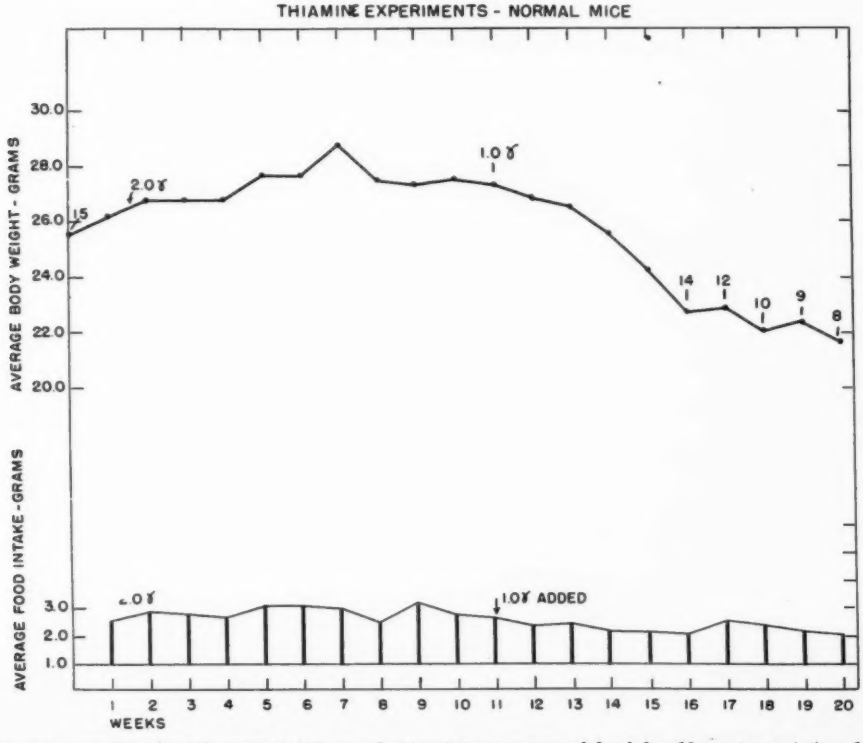

Figure 7.—Average weight of adult mice fed 2 μg. of thiamine per gram of food for 11 weeks and then 1 μg. per gram for the next 9 weeks. Lower curve, average food intake.

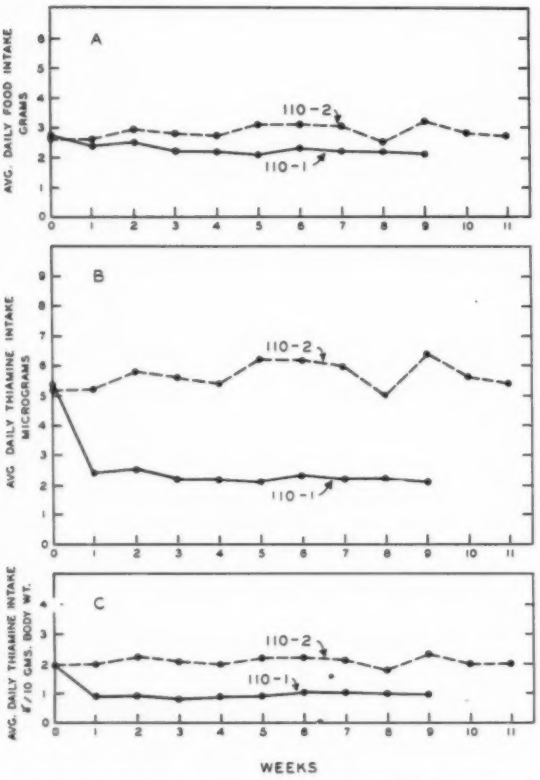

FIGURE 8.—Average daily food and thiamine intake of adult C3H mice.

of thiamine the body-weight response was different. In the slowly induced deficiency (p. —), the mice gained weight fairly rapidly for about 5 weeks and then decreased in weight at about the same rate. In acute deficiency, weight loss occurred after the first week and proceeded much more rapidly than that observed in the latter part of chronic deficiency. The experiments next described were set up to determine whether the addition of thiamine to diets of animals suffering from the chronic-deficiency syndrome would effect recovery and reverse the loss in body weight.

Two groups of mice were used. One group of 20 mice was started on low-thiamine diet 130 when they were 31 days of age. They survived on an average 48.4 days, with a range of 42–51 days. In this group, symptoms of thiamine deficiency developed between the forty-first and forty-ninth days, and the mice died 2–7 days after the appearance of the first symptoms (fig. 9, curve A).

To demonstrate the effect of thiamine supplementation following the development of symptoms of deficiency, the second group, of 11 mice, was kept on diet 130 (1 μg. thiamine per gram) until death appeared imminent. The body-weight curves of this group resembled those of the first group (fig. 9, curves A and B). At this time, the animals in the second group were given, by subcutaneous injection, 50 μg. thiamine daily with food offered ad libitum. The response was immediate and striking (fig. 9, curve C). All the animals appeared normal within 1 week. The average body weight increased 6 gm. in the first week, and 12.5 gm. in 3 weeks, or practically doubled.

PAIRED FEEDING

The production of a chronic thiamine deficiency in mice was accompanied by a decrease in food consumption, which was greater near the terminal stages, as seen in the acute thiamine-deficiency syndrome. In order to determine the degree to which starvation affected the observed decrease in body weight, mice consuming the basal diet supplemented with an insufficient amount of thiamine (1 μg. per gram of food) were compared with mice consuming the same quantities of the basal diet, but adequately supplemented with thiamine.

Twelve litter-mate pairs of 30-day-old C3H strain mice (six pairs of females and six pairs of males) were used. The experimental animals were allowed to eat low-thiamine diet 159 ad libitum, while the controls were placed on diet 161, and their food intake was kept the same as their paired

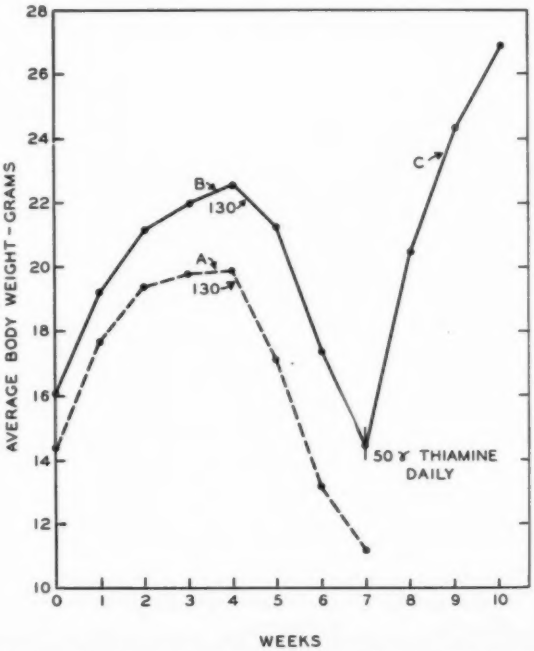

FIGURE 9.—Average weight of young C3H mice fed insufficient thiamine.

litter mates, the only difference in the two diets being in the quantity of thiamine ingested (table 1).

The average increase in body weight of the two groups was identical for the first 5 weeks (fig. 10, curves A and B). The group A mice on diet 159 began losing weight after the fifth week whereas animals on diet 161 did not begin losing weight until after the sixth week. All animals on diet 159 developed the typical thiamine-deficiency picture, while none of the control animals showed such symptoms.

Although the control animals received 10 times more thiamine, the average body weight was only slightly higher than that of the experimental animals (fig. 10, curve B compared with curve A). It appears that the progressively smaller amounts of food consumed were of greater importance in affecting body weight than was the lack of thiamine.

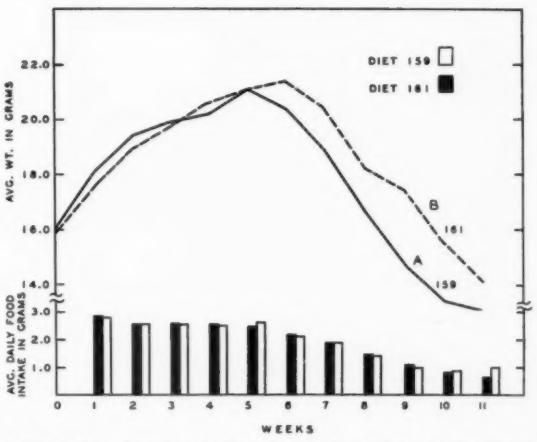

FIGURE 10.—Average body weight of 12 pairs of weanling C3H mice receiving the same quantity of food but different amounts of thiamine. Lower histograms give the average daily food intake.

DISCUSSION

Thiamine functions in the living cell combined with pyrophosphate or cocarboxylase as a coenzyme in carbohydrate metabolism. In thiamine deficiency, the metabolism of carbohydrate is incomplete, and pyruvic acid accumulates in the tissues. Diets high in fat have been shown to have a definite sparing action on the requirements for thiamine of both rats and dogs, because fat metabolism probably does not require cocarboxylase. It seems probable, therefore, that the lower thiamine requirement established for young C3H mice in this study, compared with the requirement for young albino mice determined by Hauschildt (*4*), is largely owing to the much higher fat content (20–22 percent of the diets used in these experiments compared with 3 percent fat in Hauschildt's diet).

It is believed that diets containing 20 percent fat should provide a minimum intake of not less than 2 μg. thiamine per mouse per day. Basal diets of different composition, especially in amount of fat, would require different amounts of thiamine. Diets containing lesser amounts of fat should undoubtedly contain proportionately more thiamine.

Careful consideration should be given to the vitamin intake of animals whose food consumption becomes restricted either through the effect of a deficiency of an essential constituent or through enforced restriction of food. An adequate intake of all essential constituents except the one under investigation should be insured so that an uncomplicated deficiency may be produced. Possible thiamine deficiency in particular must be avoided since in the present experiments what at first appeared to be nearly an adequate level of thiamine intake later resulted in the development of all the symptoms of acute thiamine deficiency, including the self-imposed restriction of food intake.

It is well known from pharmacologic studies that thiamine is relatively nontoxic and that vitamins in excess below the toxic level exert no effect on the healthy animal (*8*). When no exact vitamin requirements have been established for conditions of underfeeding, an excess of thiamine as well as other vitamins should be provided.

An average daily intake of 5–6 μg. thiamine (8 μg. per 10 gm. of body weight) produced a maximum growth response in young mice. An average daily intake of 3.5–4.5 μg. (2 μg. per 10 gm.) was not adequate for the maximum growth but prevented symptoms during an 8-week test period; lower levels were insufficient for growth or prevention of clinical symptoms.

Adult mice maintained their body weight for 11 weeks on an average daily thiamine intake of 5–6 μg. (2 μg. per 10 gm. of body weight). An average daily intake of 2.0–2.5 μg. (1 μg. per 10 gm. body weight) thiamine in the basal diet was not adequate for maintaining body weight. At the 1 μg. level of thiamine intake, four of nine adult animals after 7 to 14 weeks developed mild symptoms of thiamine deficiency and ultimately died.

Depletion of the initial reserves of thiamine did not hasten the appearance of thiamine-deficiency symptoms. This fact suggests that it is not the thiamine reserves per se but rather the actual daily thiamine utilization that is the important factor in the development of the deficiency syndrome. It is possible that in the process of depletion of the initial reserves the animal becomes adjusted to a more efficient utilization of thiamine.

Adult animals apparently require lesser amounts of thiamine. With an intake of 1 μg. thiamine per

10 gm. body weight, the adults survived for 9 weeks without clinical symptoms of deficiency, and only a few animals died at a later date. A similar intake (figs. 3, and 5, diet 128) by young mice resulted in marked loss in body weight, development of severe symptoms of deficiency, and death within 7 to 8 weeks.

The question arises whether the adult mouse actually requires less thiamine than the younger animals or whether the adult animal maintained first on a fairly low level of thiamine for several weeks (as in this experiment) is able to adjust its requirements to a still lower intake of the vitamin. The question cannot be answered with certainty at this time.

While both young and adult mice developed symptoms of a rapidly induced thiamine deficiency, this deficiency was more frequent in young animals, as many of the adult animals became moribund without evidencing the usual clinical manifestations of deficiency. This difference was not due to the length of survival, for both groups survived the same average number of days; nor was it due to a decreased consumption of food per unit weight by younger animals.

The rapid increase in body weight in deficient mice after supplementing the diet with thiamine and allowing food ad libitum is thought to be accounted for by an increase in the size and number of cells. Support for this belief is in the histologic observations of Dunn et al. (*7*), (1) that more mitoses were observed in the gastrointestinal mucosa and in the megakaryocytes of the spleen (a type of cell seldom seen in mitosis in the normal animal), and (2) evidence of an increase in cell volume, furnished by the increased glycogen and fat content of the liver. The increase in the adipose cells of the interacinar connective tissue of the pancreas was of similar significance.

The possibility was recognized that the muscular and testicular lesions observed in thiamine-deficient mice (*7*) may have been related to vitamin E deficiency. To obviate such a possibility, the diets used in paired feeding (diets 159 and 161) provided amounts of *dl*-α-tocopherol four times greater than those supplied in diets 126–137; and male mice were used, the growth of which is not affected by vitamin E deficiency (*10*) and which have not been observed to undergo testicular degeneration in vitamin E deficiency (Bryan and Mason (*11*), Pappenheimer (*12*)). We believe, therefore, that our diets were adequate with respect to vitamin E.

The immediate response of animals in a state of the chronic deficiency to thiamine supplementation and their complete recovery in a few days indicate that the gradual production of this syndrome in mice is due to an insufficient amount of thiamine.

Physiologic stress leads to increased activity of the pituitary and adrenal glands. This response is frequently referred to as the adaptation syndrome of Selye (*13*). In the young rat, as recently reported by Deane and Shaw (*14*), the thiamine deficiency resulted in an earlier increased stimulation of the fasciculata of the adrenal cortex than was observed with a comparable degree of inanition. Further observations of Deane and Shaw (*14*) indicated that thiamine deficiency rapidly activated the pituitary, adrenal-thymus system in the young rat, a response which appears to be entirely comparable with the adaptation syndrome of Selye (*13*).

Neither gross nor microscopic (*7*) evidence of adrenal hypertrophy was observed in either the acute or chronic thiamine-deficient C3H mice. Adrenal hypertrophy has not been observed in C3H mice.[5] Adrenal hypertrophy, however, occurs in the rat (*14*). Whether or not this species difference in adrenal hypertrophy is associated in any way with the absence in mice (*15*) and the presence in the rat of adrenal hemorrhage and necrosis (*16–20*) in pantothenic acid deficiency is not known. The relations of the function of these two endocrine glands as affected by thiamine deficiency are being further investigated.

SUMMARY

A rapidly induced thiamine deficiency was produced in both young and adult C3H female mice by the ingestion ad libitum of a thiamine-free diet. The average survival time for both groups was 24 days. Symptoms of acute thiamine deficiency developed as early as the fifteenth day, although most animals developed symptoms of deficiency between the twentieth and twenty-fifth days.

A slowly induced or chronic thiamine deficiency was produced in young mice by adding a small but insufficient amount of thiamine to the basal diet. The addition of 1 μg. of thiamine per gram of food permitted the growth of young mice for 4 to 5 weeks, following which there were loss in weight accompanied by anorexia, and the development of symptoms of thiamine deficiency which could not be distinguished from the clinical symptoms occurring in the acute syndrome.

Preliminary depletion of the thiamine reserves in young mice did not shorten the time required for the development of chronic thiamine-deficiency symptoms or decrease the rate of growth. The symptoms were completely alleviated by supplementing the diet with thiamine, and the development of the deficiency syndrome was prevented by including additional thiamine in the diet.

The average minimum thiamine intake of young mice at which no deficiency effects were noted, judged by either growth or symptoms, was between 5 and 6 μg. thiamine per mouse per day.

[5] DALTON, A. J.: Personal communication.

The minimum thiamine requirements for the maintenance of body weight of adult mice was greater than 2.5 μg. but probably less than 5 μg. to 6 μg. thiamine per mouse per day.

Addition of inositol to the basal diet in the presence of inadequate, minimal, or excessive amounts of thiamine did not affect the growth of mice.

The symptoms of hyperirritability, rigidity of legs, spinning movements, and absence of true paralysis, which develop in thiamine deficiency, are described in detail.

Enforced food restriction in the presence of adequate thiamine caused loss of weight and death within the same time as did self-induced food restriction due to the complete or partial absence of thiamine. However, symptoms of thiamine deficiency developed only in the mice deprived of thiamine.

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LESIONS OF CHRONIC THIAMINE DEFICIENCY IN MICE[1]

By Thelma B. Dunn, *pathologist*, Harold P. Morris, *nutrition chemist*, and Cel ia S. Dubnik, *chemist*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Young strain C3H mice fed a thiamine-deficient diet were observed by Morris and Dubnik (*1*) to gain in weight for approximately 10 days. After this interval, weight loss was exceedingly rapid, terminating in death of the animals in 3 to 4 weeks. Supplementation of diet with amounts of thiamine insufficient for full protection extended the survival period to 7-9 weeks. Growth occurred during the first few weeks of the supplemented diet at almost the normal rate, gradually slackened, then was followed by rapid decline in weight, loss of appetite, and the development of clinical symptoms usually between the seventh and the ninth week. These symptoms could be rapidly and completely alleviated by the addition of a sufficient quantity of thiamine. The term "chronic thiamine deficiency" has been applied to the syndrome thus produced. The composition of the diet used to produce this state of chronic thiamine deficiency in young C3H mice and a description of the principal clinical manifestations of the conditions are described in the paper by Morris and Dubnik (*1*).

The present study was undertaken to note and describe the significant lesions which develop in the course of this chronic deficiency.

MATERIALS AND METHODS

Although the pathologic changes were varied, they are, for convenience, arranged under the appropriate headings for description.

A large number of animals falling into many different groups were used. Unexpected deaths were frequent, and the final number from each group satisfactory for pathologic study was often small. From the various experiments, however, it was possible to select animals which had ingested just sufficient thiamine to permit the gradual development not only of clinical symptoms but also of definite morphologic changes. The individual groups referred to in the tables are described herein. In the discussion of the morphologic changes, no attempt was made to distinguish the individual groups since the histologic evidence of thiamine deficiency was similar in all. They are referred to as chronic thiamine deficiency, advanced stages of thiamine deficiency followed by thiamine supplementation with and without control of the amount of ingested food, controls on a thiamine-supplemented basal diet with food not restricted in amount, and pair-fed controls.

In tables 2 to 5, the groups of animals referred to are as follows:

Group 1, preliminary study (a) 4 mice, chronic deficiency and death; (b) 4 mice, recovery after thiamine supplementation on unrestricted food.

Group 2, 7 mice, thiamine-depletion period for 11–16 days, followed by a supplement of 0.8 μg.[2] of thiamine per gram of food for 57–70 days.

Group 3, 3 mice, thiamine-depletion period for 11–14 days, then a supplement of 1.0 μg. thiamine per gram of food for 66–128 days to maintain a chronic deficiency.

Group 4, 8 mice, thiamine-depletion period for 11–14 days, then a supplement of 1.0 μg. thiamine per gram of food for 35–84 days.

Group 5, 11 mice, continuous thiamine deficiency for 42–50 days.

Group 6, 11 mice, continuous thiamine deficiency for 51 days, then adequate thiamine and food ad libitum.

Group 7, 9 mice, continuous thiamine deficiency for 46 days, then adequate thiamine with restricted food for 19 days.

Group 8, 5 male mice on continuous thiamine restriction.

Group 9, 11 control mice, thiamine-depletion period for 11–16 days followed by unrestricted basal diet. Supplemented with 3 μg. thiamine per gram of food.

Group 10, pair-fed animals, food ad libitum containing 1 μg. thiamine per gram for the experimental group. The pair-fed mice received the basal diet supplemented with 10 μg. thiamine per gram of food, but the animals were restricted to the same quantity of food as that consumed by the respective deficient pair mate. The pair-fed control animal was usually killed on the same day as the experimental animal. Three controls, however, were kept until the weight loss approximated that of the experimental animals. There were 12 mice in each group.

Both the experimental and control animals were killed with ether. The experimental animals were usually killed when the clinical manifestations of thiamine deficiency appeared extreme, although a few died while under observation and were examined within an hour. No animals were accepted for histologic study in which there was an appreciable post-mortem change, although some gross pathologic lesions such as brain-stem hemorrhages and muscle degeneration were identified in animals found dead. These are included in the tables and account for apparent variation of number in the groups reported. Tissue was routinely fixed in Zenker-formol and stained with hematoxylin and eosin. Special fixatives and stains were also frequently used, as described for particular organs and tissues.

RESULTS

General Changes

A general reduction in the size of all organs was apparently due to a reduction in individual cell volume affecting both cytoplasm and nucleus. This was especially evident in the liver where the cells appeared smaller and denser, and the ratio of nuclear to cytoplasmic material appeared to be increased. There seemed to be no corresponding re-

[1] Received for publication August 5, 1947.

[2] The abbreviation μg. is used herein instead of the word "microgram."

duction in size of the vascular bed, and as a consequence the volume of blood in many organs, notably lungs, liver, intestine, brain, bone marrow, the mesentery, and subcutaneous connective tissue, appeared markedly increased. All fat was greatly reduced, and the tissues when handled at autopsy appeared sticky and dehydrated. In table 1 the weight of the heart, spleen, and brain of the mice in the pair-fed groups are given, together with weights of stock strain C3H mice of the same age on unrestricted food.

TABLE 1.—*Body and organ weights of experimental chronic thiamine-deficient mice compared with weights of pair-fed controls and stock strain C3H mice of similar age*

Mice	Number	Weight of—			
		Body (Gm.)	Heart (Mg.)	Spleen (Mg.)	Brain (Mg.)
Thiamine-deficient males	6				
Range		10.6–15.3	72.8–93	16–20.6	461–552
Average		13.3	80.1	16.7	510.8
Percentage of body weight			.64	.13	3.9
Thiamine-deficient females	6				
Range		10.7–13.1	65–101	12–20	521–558
Average		11.9	76.9	16.6	532.5
Percentage of body weight			.65	.14	4.3
Average percentage of body weight (males and females)			.65	.14	4.1
Pair-fed control males	6				
Range		13.0–18.3	63.4–88.8	13–60	497–641
Average		15.9	74.6	34.1	550
Percentage of body weight			.48	.21	3.6
Pair-fed control females	6				
Range		10.6–15.9	63.2–82.6	10–48.6	482–634
Average		12.7	73.2	24.7	547.7
Percentage of body weight			.54	.18	4.0
Average percentage of body weight (males and females)			.51	.19	3.8
Stock C3H males, food ad libitum, age 9 weeks	20				
Range		23–30	98–128	83.9–184.6	484.2–598
Average		26.4	110.2	106.1	538.6
Percentage of body weight			.42	.40	2.0
Stock C3H females, food ad libitum, age 14 weeks	20				
Range		21–26.3	88–110	105–173	441–514
Average		24.6	101.7	138.8	486
Percentage of body weight			.41	.56	1.97
Average percentage of body weight (males and females)			.41	.48	1.98

In the thiamine-deficient animals the proportion of heart weight to body weight was higher than in the pair-fed controls, which in turn was higher than in stock strain C3H mice of nearly the same age receiving food ad libitum. The figures indicate that although the actual heart size in thiamine deficiency and in food restriction was reduced below that of the normal control, the proportionate size to body weight was considerably increased; but actual cardiac enlargement such as is reported in human beings with beriberi was not found. The spleen, on the other hand, was actually and proportionately smaller in the thiamine-deficient and food-restricted animals. The brain, like the heart, maintained a greater proportional weight, similar in the deficient and in the food-restricted animals. Such findings are consistent with the opinion that the most essential organs are longest spared in certain inanition states and that the lymphoid structures are particularly labile during food restriction.

NERVOUS SYSTEM

Many previous reports on vitamin B deficiency were concerned with changes described in the peripheral nerves and central nervous system of laboratory animals. These changes were reviewed by Prickett (*2*), and by Davison and Stone (*3*). Because of these reports, special investigations were made on the nervous tissue of the deficient mice, particularly in the early periods of the experiment, before it was learned that demonstrable injury was largely restricted to the brain stem. These early investigations were carried out on (1) myoneural end organ, (2) sciatic nerve, (3) the spinal cord, and (4) the brain. Because of the frequency of changes in the brain, this organ was examined in all groups of mice (table 2).

Examination of nerve tissue was generally unsatisfactory because of the technical uncertainties encountered. Only by close comparison with the

TABLE 2.—*Nervous system of C3H mice in thiamine deficiency*

Group [1]	Number of mice	Sex	Myoneural end organ [2]	Sciatic nerve [2]	Spinal cord [2]	Brain hemorrhage [2]	Clinical manifestations
1a (D)	4	F	2−	4−	3−	4+	Incoordination weakness.
1b (R)	5	F	1−	4−	0	5−	Normal.
2 (D)	7	F	3−	3−	6−	6+	Incoordination.
3 (D)	4	F	2−	1−	3−	1+ 1+ 2+	Do.
4 (D)	8	F	2−	5−	6−	2+ 1+	Do.
5 (D)	10	F	0	0	0	6+ 8+ 2−	Do.
6 (DR)	11	F	0	0	0	[7] 11−	Normal.
7 (DCR)	9	F	0	0	0	9−	Do.
8 (D)	5	M	0	0	0	2+	Incoordination.
9 (control)	11	F	0	2−	9−	[4] 3− 11−	Normal.
10 (pair-fed)	[5] 6	F	0	0	0	3+ 3− 5+	Do.
	[5] 6	M	0	0	0	1− 1+	Normal.
	[6] 6	F	0	0	0	5− 6−	Do.
	[6] 6	M	0	0	0		

[1] Explanation of letters in parentheses: D, chronic thiamine deficiency; R, recovery with adequate thiamine; DR, recovery with adequate thiamine and food ad libitum; DCR, recovery with adequate thiamine and food restriction.
[2] 0, no examination; − examined, no lesion; +, examined, lesion present.
[3] 1 with macrophages containing brown pigment.
[4] 1 with gliosis.
[5] Experimental.
[6] Control.

control animals could any conclusions be hazarded in regard to the significance of many conditions observed. Vacuolation usually developed in the brain-stem and spinal cord sections, particularly in the gray matter, in both experimental and control animals when the tissues were fixed in formalin. The Marchi preparations showed variable and inconsistent blackening. Moreover, nerve ganglion cells with shrunken and deeply staining nuclei were frequently seen in all groups of animals. Results such as these may account for the contradictory conclusions reached by various investigators on the effects of thiamine deficiency on the nervous system of certain laboratory animals.

The myoneural end organs were examined in 10 deficient animals. Telford's (*4*) modification of Bodian's method was used. No degenerative changes were found in the end organs; and even in one preparation where muscle degeneration had developed, the end organs appeared uninjured. Because of these consistently negative findings, no further study of the end organs was made, and it was concluded that this was not a site of nerve-tissue damage in thiamine deficiency in the mouse under the conditions of this experiment.

A section of sciatic nerve was examined from 13 deficient animals. No degeneration was observed, a finding in agreement with the findings of Berry, Neumann, and Hinsey (*5*) in the cat, Wintrobe et al. (*6*) in the pig, and Prickett (*2*) in the rat.[3] In addition to the Weil and Marchi method, the technique of Covell and O'Leary (*8*), a vital staining method in which neutral red is applied to the nerve removed immediately after death, was also used to examine six nerve sections. As controls, sciatic-nerve sections from normal mice and from rats in which the nerve has been cut proximally 3–7 days previously were used. While degenerative changes were strikingly evident in the surgically severed nerves, no similar changes were found in the thiamine-deficient or normal animals.

Spinal cord sections from 18 deficient and control animals were examined at 3 levels, cervical, thoracic, and lumbar, by the Marchi and Weil methods. No focal degenerative changes could be reliably identified, although the Marchi method showed areas of blackening as frequent in the control as in the experimental animals and not related to any special fiber tracts.

The head of each experimental and control animal was severed from the body at autopsy, the galea was reflected, and the skull cap was removed. They were then fixed in situ in the skull base in 10-percent formalin for 5–10 days to avoid the artificial production of extravasation of blood by pulling and bending the very soft and friable fresh brain. Hemorrhages in the brain stem were observed in 30 deficient mice of a total of 50, and in 1 control animal which belonged to the pair-fed group. It is reasonably certain that these hemorrhages were not post-mortem artifacts. They were usually observed in the intact brain as darkened areas showing through a thin layer of nervous tissue. They were found in the medulla, in the area lying below the trapezoid body, above the roots of the hypoglossal nerve, and medial to the roots of the glossopharyngeal and vagus nerves (fig. 1) and appeared most extensive at the level of the junction to the pons. If the cerebellum was lifted, dark areas could also be seen below the floor of the fourth ventricle. On section at this level, these hemorrhages were seen to be bilateral, both sides usually appearing equally affected. They tended to occur in the dorsal half of the medulla just below the floor of the fourth ventricle and thereby were localized chiefly in the reticular substance. They also appeared dorsolaterally in the general region of the vestibular nuclei. However, no definite, consistently recurring localization in respect to any brain-stem nuclei could be made out. Numerous sections were made of other portions of the central nervous system, but no hemorrhages were found in the spinal cord, the cerebellum, or the end brain, except in 1 partially deficient animal in the pair-fed group, in which small hemorrhages were found adjacent to the third ventricle.

FIGURE 1.—Longitudinal section of brain showing location of hemorrhagic areas.

Sections from the hemorrhagic areas were stained by the Marchi, Weil, Nissl, and hematoxylin-and-eosin methods (fig. 2, *A*, *B*, *C*), and a few slices of whole brains were cleared in glycerin. This last procedure proved a useful method for showing the extent of the hemorrhage. On

[3] In a later study using polarized light, Prickett, Salmon, and Schrader (7) observed changes of an edematous type in the peripheral nerves which were not shown by sudan III.

microscopic examination, masses of well-preserved erythrocytes were seen which appeared to lie free in the nervous tissue (fig. 3, *A*).

Evidence of the ante-mortem existence of these hemorrhages was indicated, however, by the occasional presence of a small irregular area of homogeneous, pink, coagulation necrosis in the center, sometimes containing one or two pyknotic nuclei (fig. 3, *B*). These centers of necrosis and surrounding hemorrhage resembled the small "ring"

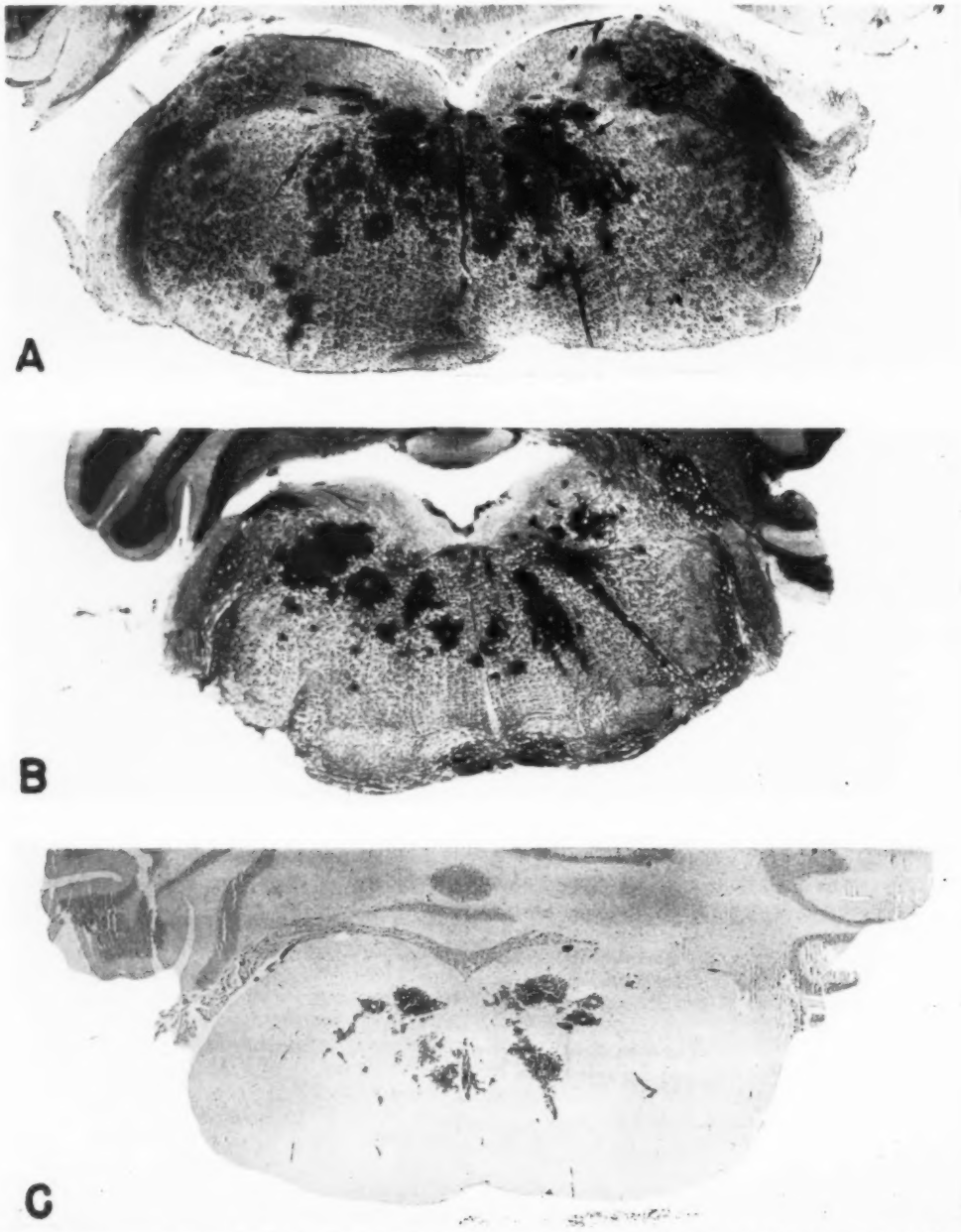

FIGURE 2.—Transverse sections at level of pons and medulla, showing hemorrhages. *A*, Marchi stain. ×20. *B*, Weil stain. ×20. *C*, Hematoxylin-and-eosin stain. ×20.

hemorrhages described in the human brain in a variety of pathologic conditions (Schaller, Tamaki, and Newman (*9*), Baker (*10*)). Acute swelling of oligodendroglia was observed in the hemorrhage area about the fourth ventricle and to a lesser degree in some pair-fed control animals, while it was absent from the animals with normal food intake.

Vessels adjacent to these areas of hemorrhage were widely dilated and stood out prominently in the sections cleared in glycerin. These glycerin-cleared sections sometimes also showed small masses of extra-vascular erythrocytes along the course of the vessels. No evidence of endothelial proliferation was seen in these areas.

In only one brain, where a few macrophages containing blood pigment were seen, was evidence of preceding old hemorrhage found. In another continuously deficient animal, a small area of gliosis was found (fig. 3, *C*). On the basis of these findings, it seems probable that these hemorrhages developed acutely and terminally and usually led to the death of the animal.

Clinical manifestations could not be correlated with the appearance of hemorrhage, since animals were usually killed when they appeared moribund, and there appeared to be the same degree of weakness and incoordination in animals with and without brain-stem hemorrhage. True paralysis did not develop.

Nerve-Tissue Changes

Hemorrhagic lesions in the brain arising after vitamin B deficiency were described in rats (Prickett (*2*)), in foxes, in which it is known as Chastek paralysis (Evans, Carlson, and Green (*11*)), and in pigeons (Alexander et al. (*12*)). Several authors (Alexander (*13*), Kravitz and Stockfisch (*14*), Zimmerman (*15*), and De Wardener and Lennox (*16*)) considered that Wernicke's disease in man is of a similar nature.

In the rat, Prickett (*2*) reported that disseminated foci of hemorrhage in the brain or intense congestion of one or both sides were found to involve Dieter's nucleus, the chief vestibular nucleus, Bechterew's nucleus, and the nucleus solitarius in approximately 75 percent of the animals in the deficient group. Prickett's study also included peripheral nerves, in which with the staining methods used he found no evidence of degeneration.

It may be pertinent to this discussion that Craigie (*17*), working with the rat, measured the capillary bed of various parts of the brain and concluded that the gray matter is more richly supplied than the white matter, and the sensory and correlation centers than the motor nuclei. The richest center observed in the rat was the dorsal cochlear nucleus, followed by the chief vestibular nucleus, the nucleus dentatus, and the granule layer of the cerebellar cortex. These areas correspond closely to the sites of hemorrhage described by Prickett in the rat. In Chastek paralysis of foxes, described by Evans, Carlson, and Green, the lesions are found only in the gray matter. This report of the condition in foxes also emphasized a "striking proliferation of the endothelium and of the adventitia of small vessels" in focal areas. Small parenchymal hemorrhages were considered secondary to the degenerative vascular changes. The authors also observed that "hemorrhages are common in the reticular formation of the medulla, but proliferative lesions do not occur there." Chastek paralysis in foxes is considered by Evans, Carlson, and Green to be the pathologic counterpart of Wernicke's hemorrhagic polioencephalitis in chronic alcoholism in man. Alexander (*13*) compared the lesions of Wernicke's disease with those of experimentally produced B_1 avitaminosis of pigeons and considered them identical in many respects. Alexander concluded that—

> angiodegeneration with varicose deformity of the vascular bed is the primary change in Wernicke's disease, and the resulting subacute necrosis of the parenchyma with glial proliferation and proliferation of endothelial and adventitial elements of the vascular walls as well as the hemorrhages are secondary.

The central-nervous-system lesions of the mouse are similar in general to those of other species. However, there are certain important differences. No proliferative vascular change was observed in the mouse. The hemorrhages apparently developed along the course of greatly dilated vessels. The reticular formation in the medulla was the area most frequently and severely involved, and no localization to any particular nuclei was observed. Nerve-cell damage was minimal, and little evidence of recovery from the acute condition was found. It may be that the rich vascular supply of the reticular formation in the mouse makes this site particularly vulnerable to hemorrhage during prolonged partial thiamine deficiency. Since this area is so closely connected with respiration and various vegetative and reflex activities (Tilney and Riley (*18*)), it seems probable that extensive hemorrhage at this site proved rapidly fatal, and consequently little or no nerve cell damage, vascular proliferation, or gliosis developed. No report of the relative vascularity of different areas in the mouse brain could be found in the literature; but a species variation probably exists, which may account for differences in the localization of what appears to be essentially the same hemorrhagic process found in thiamine deficiency in the different species studied. These findings for the mouse under the conditions of this experiment support the opinion of Engel and Phillips (*19*), who studied chicks and rats. They

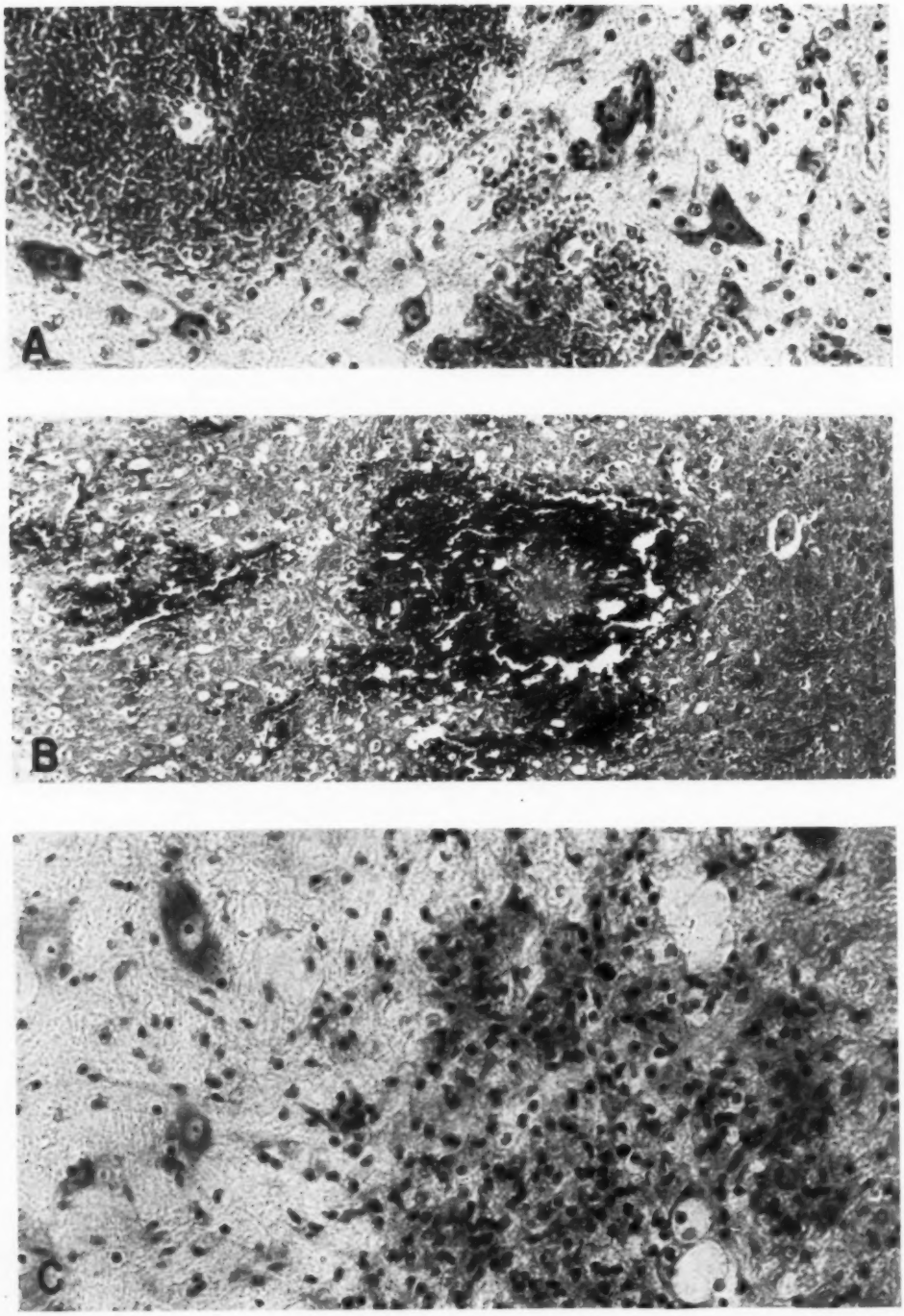

FIGURE 3.—Details of lesions in the brain-stem. *A*, Hemorrhage and ganglion cells. Nissl stain. ×350. *B*, Hemorrhage with central necrosis. Hematoxylin-and-eosin stain. ×100. *C*, Area of gliosis. Nissl stain. ×350.

wrote that their findings demonstrated that "the nervous symptoms of uncomplicated experimental beri-beri are not due to any definite anatomical change in the nervous system." It is our conclusion that the hemorrhagic lesions in the brain-stem are not necessarily caused by a preceding degenerative process in the nerve tissue or degeneration of the vessel wall. It is suggested that an increased capillary fragility (which may also account for the gastrointestinal hemorrhages) in a highly vascular area such as this portion of the brain, where the vessel walls have little anatomic support, leads to this hemorrhagic lesion.

Reproductive System

The female reproductive organs were reduced in size in all thiamine-deficient groups. In the ovary there was a marked reduction in the number of corpora lutea. Indeed, in the most severely affected ovaries no luteal tissue could be recognized. While follicles in the earliest stages of development were abundant and a few medium-sized follicles were usually present (fig. 4), the number of atretic follicles appeared to be increased. Many "wheel cells" were present in the medulla. When the animals were given food ad libitum with adequate thiamine, they recovered rapidly, and the ovaries became essentially normal. The ovaries of animals given adequate thiamine but remaining on restricted food intake showed no recognizable difference from those of the continuously deficient animals. The size of the uterus was reduced, and endometrial glands were often lacking in the section. The vaginal epithelium failed to show a cycle variation and often appeared atrophic, or there might be an infiltration of the vaginal mucosa with leukocytes, as in the diestrus condition. In some animals, the vagina was distended with a mucoid fluid containing many degenerating polymorphonuclear leukocytes and cellular debris. The calorie-restricted animals in the pair-fed group showed a similar diestrus or atrophic condition of the vagina.

These histologic appearances in the ovaries of thiamine-deficient and calorie-restricted mice corresponded to the condition in calorie-restricted mice described by Huseby and Ball (*20*). Other authors, among them Marrian and Parkes (*21*) and Mulinos and Pomerantz (*22*), are of the opinion that thiamine deficiency with inanition and inanition alone produce identical changes in the ovary, and that these in turn are secondary to the failure of normal activity of the hypophysis since a similar genital atrophy occurs after hypophysectomy.

Most of our observations on thiamine deficiency were made on female mice (table 3); but after testicular damage was observed in a single male in one of the deficient groups, a special group of males was studied (group 8). Males were also included in the pair-fed group. On gross examination, the testes in the thiamine-deficient mice were always reduced in size and retracted into the abdomen. Microscopically, various degrees of damage were observed. In earlier stages, the tubules of the epididymis contained many nucleated cells (spermatids and spermatocytes); but in more advanced degeneration, the epididymis was usually empty of sperm and cells. As is frequent in degenerative conditions of the testis, the tubules in many specimens appeared unequally involved (fig. 5, *A* and *B*). In several mice, however, the tubules were remarkably uniform, since no spermatoza were found; and the lumina were solidly filled with nucleated cells, identified as primary spermatocytes (fig. 5, *C*), which were also abundant in the tubules of the epididymis. Multinucleate giant cells were infrequent and never con-

Figure 4.—Effect of thiamine deficiency on the ovary. *A*, Atrophy with absence of corpora lutea in ovary of deficient animal. *B*, Ovary of control animal. Hematoxylin-and-eosin stain. ×10.

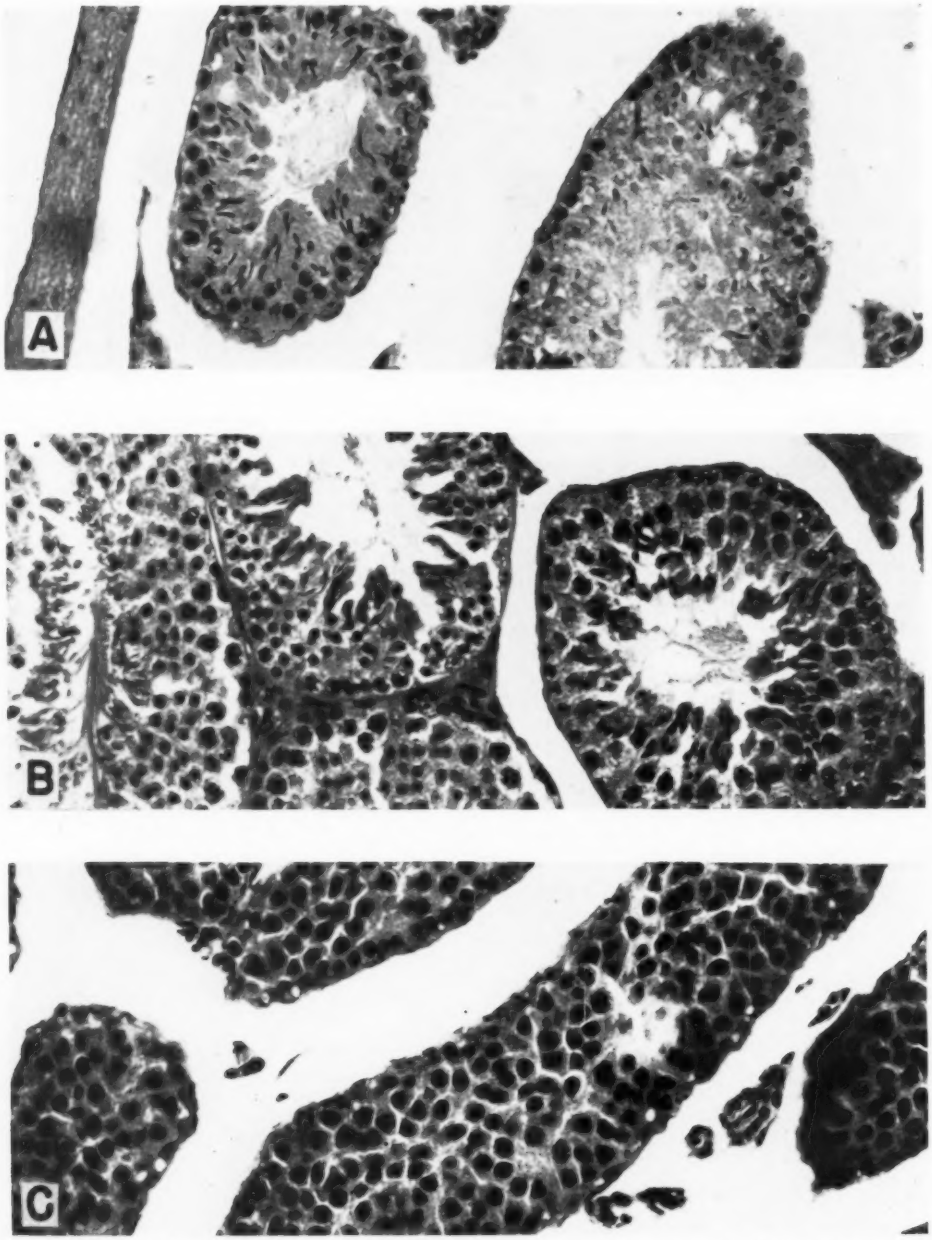

FIGURE 5.—Testis changes in thiamine deficiency. *A*, Tubules showing varying degrees of loss of spermatogenic cells. *B*, Tubules from a control mouse. *C*, Tubules solidly filled with nucleated cells. Hematoxylin-and-eosin stain. ×350.

tained more than three or four nuclei. In the pair-fed thiamine-deficient group, the picture was especially uniform and suggested that the maturation of spermatozoa had failed, since neither these cells nor spermatids were seen, and the predominant cell form was a spermatocyte. Sertoli cells usually appeared unaffected, although occasional tubules contained such cells free in the lumen. The

TABLE 3.—*Effect of thiamine deficiency on reproductive organs of C3H mice*

FEMALES

Group [1]	Number of mice	Ovaries		Uterus		Vagina		Comments on groups as a whole
		Number examined	Corpora lutea	Number examined	Condition	Number examined	Condition	
1a (D)	4	3	3+	4	Slight atrophy in all.	4	2, atrophy and debris; 2, atrophy.	No essential difference in these 2 groups.
1b (R)	5	5	5+	3	4, normal	3	3, atrophy	Reduced amount of luteal tissue.
2 (D)	6	5	5+	6	5, normal; 1, atrophy.	6	6, atrophy [2]	Differ from preceding in degree; more advanced.
3 (D)	4	0		0		2	1, extreme atrophy; 1, slight atrophy.	
4 (D)	8	7	4− 3 reduced	6	6, atrophy	6	4, atrophy; 2 atrophy and debris.	Atrophy more advanced than in preceding groups.
5 (D)	10	3	1+ 2 reduced	5	5, atrophy	9	5, proestrus state, atrophy; 4, atrophy or diestrus.	Generally atrophic, but evidence of cycle variation.
6 (DR)	11	7	6 normal 1 reduced	10	10, normal	11	10, normal; 1, atrophy	Essentially normal, follicular fluid present, possibly increased above normal.
7 (DCR)	6	4	3 absent 1 reduced	4	4, atrophy	5	Atrophy in all	No essential difference in this group and thiamine-deficient group. May be slightly less luteal tissue, fewer atretic follicles and more severe atrophy of vagina.
9 (control)	11	8	Normal	11	Normal	11	Normal	
10 (pair-fed)	[3] 6	6	6 reduced	6		5	Atrophy and diestrus in all	
	[4] 6	6	5 reduced 1 normal	6		5	2, atrophy; 3, atrophy and diestrus.	No essential difference in the 2 groups.

MALES

		Testes—Condition	
8 (restricted)	6	6	2 showed spermatogenesis, apparently not proceeding beyond spermatocyte stage. 4 showed reduced spermatogenesis, varying in tubules; some sperm in epididymis.
10 (pair-fed)	[3] 6	6	All showed failure of spermatogenesis, which appeared to be arrested at stage of primary spermatocyte.
	[4] 6	6	Normal spermatogenesis.

[1] For explanation of letters in parentheses, see footnote 1, table 2.
[2] 3 with debris.
[3] Experimental.
[4] Control.

interstitial cells appeared to be numerically normal but small in size with deeply staining, nonvacuolated cytoplasm. The pair-fed control animals on calorie restriction had apparently normal spermatogenesis, and spermatozoa were abundant in the epididymis.

No published report of testicular damage following thiamine deficiency has been found for any other mammalian species. Marrian and Parkes (*23*) described testicular changes in vitamin B deficiency in the pigeon and believed that the changes were not necessarily a starvation effect, since birds dying from inanition alone failed to show degeneration as severe as that in the vitamin-deficient group. They also emphasized the similarity of the degenerative changes which may be caused in the testis by a wide range of adverse conditions and considered that the picture of vitamin B_1 deficiency is extraordinarily reminiscent of the testis following exposure to X-rays and also characteristic of the condition due to nondescent or vasectomy.

The effect of inanition on the testis of the adult rat was described by Siperstein (*24*). In this species, degenerative changes were observed in a few irregularly scattered tubules. The process involved first the spermatids and spermatozoa, then the secondary and primary spermatocytes, and finally the spermatogonia. The Sertoli cells were the most resistant. During the degenerative process, "multinucleated giant cells of 3-4 nuclei arose, apparently by fusion of the degenerating spermatocyctes." No hypertrophy of the interstitial tissue was found accompanying atrophy of the seminiferous epithelium during inanition in either young or adult rats.

It would be desirable to exclude the possibility that the testis changes in the thiamine-deficient mice were not caused by inanition or a possible vitamin E deficiency. The apparently normal condition of the testes in the pair-fed control males indicates that inanition alone is not the factor, and the excessive amounts of vitamin E given to these pair-fed experimental animals makes it highly improbable that deficiency in this vitamin is responsible. Pappenheimer (*25*) and Bryan and Mason (*26*) agreed that the male mouse, unlike the rat, fails to develop testicular degeneration and sterility on diets low in vitamin E. In the case of vitamin E deficiency in the rat, Mason (*27*) pointed out that different conditions exist in different tubules with clumping of mature sperm in the early stages, and formation of multinucleate giant cells with 2 to 40 degenerating, spermatid nuclei later. While there was no unique histologic picture in the testes of our male mice on a thiamine-

deficient diet that would distinguish them from the other types of testicular degeneration just reviewed, the absence of multinucleate cells makes the histologic picture unlike the description of the condition of the testes in vitamin E deficient rats.

Muscular System

Degenerative lesions were found in skeletal muscles with great frequency in 24 of 63 partial-thiamine-deficient animals (table 4). In the pair-fed group, 7 of the 12 experimental and 3 of the 12 control animals showed the muscle lesion. All 3 of these pair-fed control animals were extremely emaciated, weighing 10.6, 11.2, and 13 gm., respectively. Histologically, the muscle lesions closely resemble changes described in vitamin E deficiency, except that what may be considered an acute stage with edema and neutrophilic infiltration was observed only once. The muscle degeneration was first observed on gross examination of a mouse in a recovery stage receiving supplemental thiamine. The animal's movements were active and normal; but when the skin was reflected, numerous fine silvery streaks were seen in practically all muscles. Following this observation, all animals were examined for similar changes. The muscles most frequently involved were the rectus capitis group (fig. 6) and the longissimus dorsi. Often these were the only muscle groups in which the lesion was found grossly; and even when sections were taken directly from these muscles, single sections sometimes failed to include the degenerated areas. On the other hand, routine microscopic sections of vertebral marrow and attached muscles and sections of thyroid and attached muscles sometimes showed a few degenerated fibers. This muscle degeneration, therefore, was probably more frequent and widespread than our figures indicate, since it was probably overlooked in some mice.

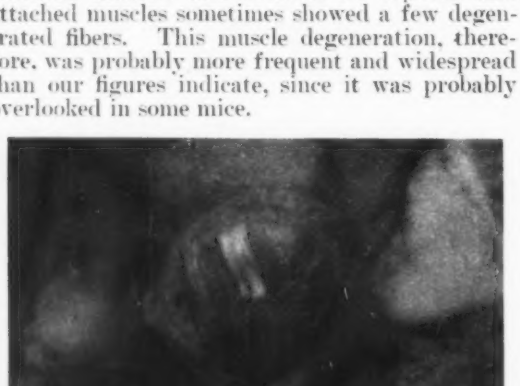

Figure 6.—Muscle degeneration with silvery streaks in rectus capitis muscle group.

Table 4.—*Muscle degeneration in thiamine-deficient C3H mice*

Group [1]	Number of animals	Muscle degeneration [2]		
		In skeletal muscle	Myocardial	Colon
1a (D)	4	0	0	.....
1b (R)	5	2	1	.....
2 (D)	7	1	0	.....
3 (D)	4	1	3	.....
4 (D)	8	1	2	.....
5 (D)	10	3	0	.....
6 (DR)	11	4	.....	.....
7 (DCR)	9	[3] 9	.....	1
8 (R)	5	3	1	.....
9 (control)	11	0	.....	.....
10 (pair-fed)	[4] 6	2	([5])	.....
	[5] 6	4	([5])	.....
	[7] 6	1	([5])	.....
	[8] 6	3	([5])	.....

[1] For explanation of letters in parentheses, see footnote 1, table 2.
[2] Increasing incidence in later groups probably due to more careful search with dissecting microscope.
[3] 1 tongue.
[4] Control females.
[5] Not examined.
[6] Experimental females.
[7] Control males.
[8] Experimental males.

Microscopically, it was most frequently seen as a finely granular swollen area in the fiber (fig. 7, *A*), often with early calcific deposition surrounded by cells with indistinct peripheral margins (fig. 7, *B*) which were often coherent and similar to lesions described in rats receiving sulfaguanidine, as described by Ashburn et al. (*28*). This lesion was also similar to changes in mice in vitamin E deficiency described by Pappenheimer (*25*).

Although the majority of hearts from deficient animals appeared normal, a few showed degeneration of the myocardium. Spontaneous degeneration of the myocardium is not uncommon in our strain C3H mice. It consists of areas of hyaline necrosis, followed by fibrosis and extensive calcification usually located just below the epicardial surface at the base of the ventricles. The spontaneous lesion is seldom accompanied by any evident cellular reaction. In two hearts from experimental mice, however, a different type of change was found, which consisted of areas of granular degeneration in the ventricular septum (fig. 8), and the walls of the auricle showed a condition similar to but less severe than that described by Ashburn and Lowry (*29*) for rats on thiamine deficiency. Several other hearts showed less extensive degeneration, localized in the ventricular septum and apex of the heart. It seems probable that these myocardial changes are directly related to the deficient diet and that mice as well as rats (*29*) and pigs (*30*) may develop cardiac changes with thiamine deficiency.

Degenerative changes were found in the muscle of the tongue in two instances and once in the smooth muscle from the wall of the colon. Pappenheimer (*24*) stated that the tongue and smooth

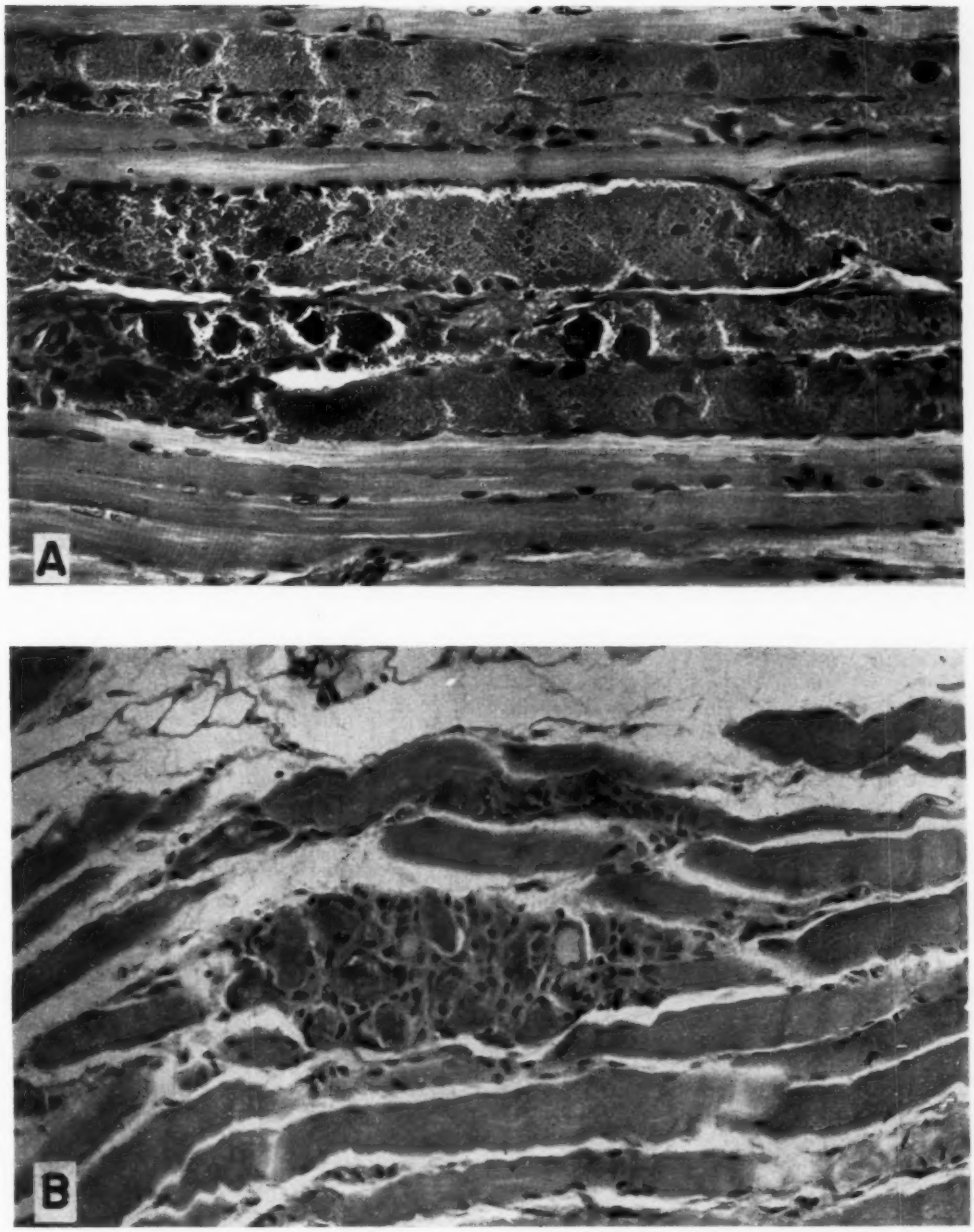

FIGURE 7.—Muscle degeneration. *A*, Granular substance with patches of early calcification replacing muscle fibers. *B*, Syncytiallike cell masses surrounding necrotic muscle tissue. Hematoxylin-and-eosin stain. ×285.

muscle have not been found affected in vitamin E deficiency.

HEMATOPOIETIC AND LYMPHATIC ORGANS

Atrophy of the lymphatic and blood-forming organs was extreme (table 5) and disproportionate to that occurring in other organs, as can be seen from the spleen weights (table 1). Histologically, there was a reduction in all cellular elements in the spleen, particularly in small lymphocytes. Extramedullary hematopoiesis, which was found regularly in the spleens of control animals at this age,

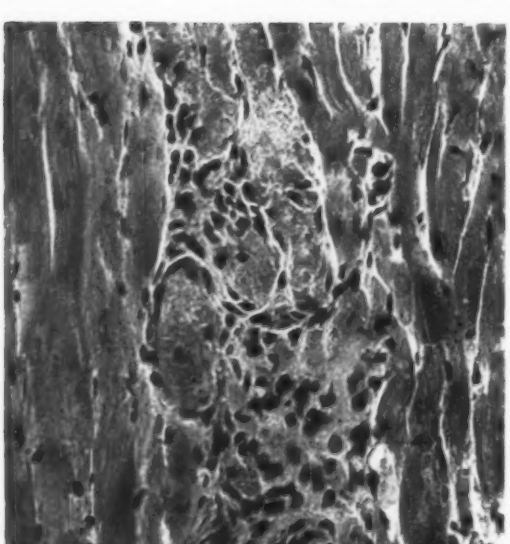

FIGURE 8.—Myocardial degeneration. Granular necrotic masses of tissue in ventricular septum. Hematoxylin-and-eosin stain. ×285.

was absent. The trabeculae and vessel walls were very prominent, and there was an apparent increase in phagocytic cells containing dark brown pigment in the splenic pulp. The number of erythrocytes within the vessels and pulp appeared much reduced, and there was no vascular dilatation such as appeared in many other organs (fig. 9). In animals in a recovery stage receiving thiamine and food ad libitum, there was extreme hematopoietic activity in the pulp, frequent eosinophils, and numerous mitoses of megakaryocytes, but relatively little germinal activity in the follicles in comparison with control mice receiving food ad libitum. The pair-fed controls were similar to the deficient animals.

The bone marrow appeared congested. The vascular spaces were widely dilated and filled with erythrocytes. The cellular marrow was much reduced in area, and myelocytic types seemed to predominate (fig. 10). On recovery, the cellular areas were greatly increased, and myelocytic types and eosinophils remained more abundant than erythropoietic forms.

The lymph nodes were reduced in size, and many showed small and large fluid-filled spaces in the medulla. The number of small lymphocytes appeared to be greatly reduced. Many large monocytic phagocytes with eosinophilic cytoplasm sometimes containing pigment were seen in the sinuses of occasional nodes. The lymphocytic cell nuclei generally appeared pyknotic and occasionally were fragmented and ingested by phagocytes (fig. 11, *A*). On recovery, the lymph nodes showed more active reaction centers than were seen in control animals.

The thymus showed probably the most extreme degree of atrophy. Often the lymphocytes were relatively more abundant in the medulla than in the cortex or had almost completely disappeared, and only thymus epithelial cells remained (fig. 11, *B*). Recovery appeared to be rapid and complete on thiamine supplementation and unrestricted diet.

The histologic evidence of recovery was not uni-

TABLE 5.—*Changes in reticulo-endothelial system of thiamine-deficient C3H mice* [1]

Group	Number of mice	Sex	Spleen				Bone marrow			Lymph nodes		Thymus	
			Number examined	Atrophy	Extramedullary hematopoiesis	Megakaryocyte mitosis	Number examined	Atrophy and congestion	Myelocytic cells predominant	Number examined	Atrophy	Number examined	Reduced lymphocytes
1a (D)	4	F	4	4+	4−	4−	2	2+	2+	2	2+	0	----------
1b (R)	5	F	5	2+	2−	1+	4	1+	3+	0	----------	0	----------
				3−	3+			3−					
2 (D)	7	F	7	7+	7−	7−	3	3+	3+	7	7+	2	1
													1−
3 (D)	4	F	2	1+	1+	1+	3	2+	2+	1	1+	0	----------
				1−	1−	1−		1−	1−				
4 (D)	8	F	7	7+	1+	7−	3	3+	[2] 3+	6	6+	1	1+
					6−								
5 (D)	10	F	9	9+	9−	9−	9	9+	9+	8	8+	4	4+
6 (DR)	11	F	9	9−	9+	9+	5	5−	5+	9	9−	4	1+
													3−
7 (DCR)	9	F	7	3+	3+	3+	3	1+	1+	5	2+	0	----------
				4−	4−	4−		2−	1−		3−		
8 (D)	5	M	3	3+	3−	3−	2	2+	2+	2	2+	1	1+
9 (control)	11	F	10	10−	10+	5	5	5−	5−	5	5−	2	2+
10 (pair-fed)	[3] 6	F	6	6+	6−	6−	2	2+	2+	0	----------	2	2+
	[4] 6	F	4	4+	4−	4−	2	2+	2+	0	----------	1	1+
	[3] 6	M	3	3+	3−	3−	2	2+	2+	1	1+	----------	----------
	[4] 6	M	3	3+	3−	3−	1	1+	1+	0	----------	1	1+

[1] For explanation of letters in parenthesis, see footnote 1, table 2.
[2] 1 slight erythropoieses.
[3] Experimental.
[4] Control.

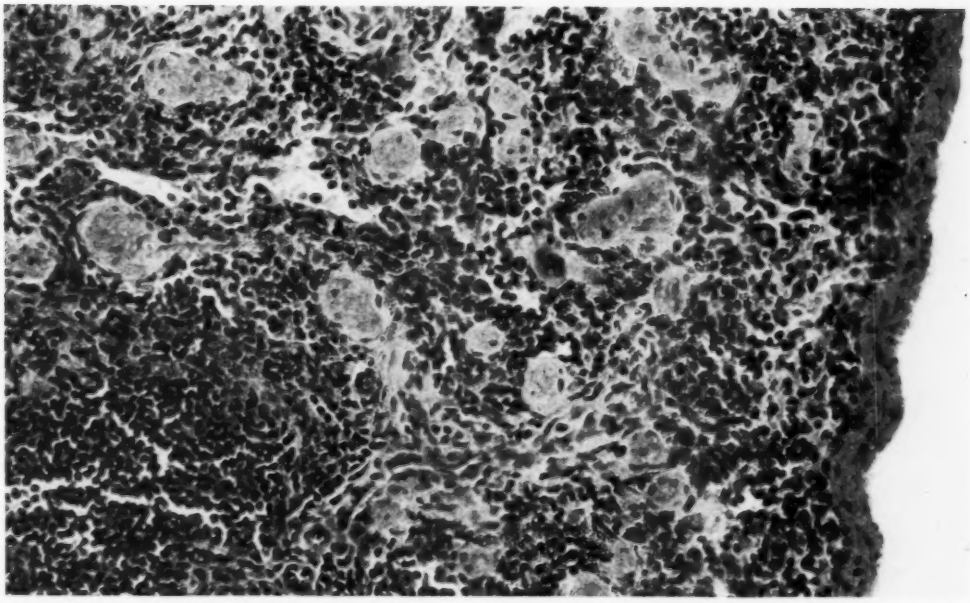

FIGURE 9.—Spleen. Atrophy, reduction in lymphocytes, and apparent increase in trabecular and capsular connective tissue. Hematoxylin-and-eosin stain. ×350.

form in animals given thiamine but restricted as to food intake. Some showed active hematopoiesis, while others resembled the animals with continuing thiamine deficiency.

In summary, all blood-forming and lymphatic tissues showed atrophy, the greatest reduction in number being in the lymphocytic cells.

In pair-fed groups no histologic differences were detected between the thiamine-deficient and food-restricted animals. This is in accordance with the observation of other workers that all the changes in the hematopoietic organs described in thiamine deficiency are also found with calorie restriction. A recent report on this subject of calorie restriction in the rat was made by Andreasen (*31*), who concluded that the most severe relative weight loss in inanition was in the liver, lymph nodes, spleen, thymus, and adipose tissue, and that the atrophy of the thymus and fat depots may be so pronounced as to approach a complete loss of the specific tissue. The relative loss of lymphocytes, especially in young animals, indicated to him that substances of importance to the growth of the individual are deposited in the lymphocytes.

MISCELLANEOUS ORGANS AND TISSUES

Observations on this group of animals revealed a generalized cellular atrophy in various organs and tissues other than those previously described, and apparent congestion of the vessels. This was the only change observed in the lungs and kidneys. When the liver was stained for fat, a complete absence of sudanophilic material was revealed in nearly all the thiamine-deficient animals. In animals receiving thiamine but restricted calories, small globules of fat were found. When thiamine-deficient animals, after thiamine supplementation, were allowed food ad libitum, an excessive amount of fat, frequently in rather large globules, was present in comparison with unsupplemented controls. Glycogen was found in the livers of deficient animals, but usually in much reduced amounts in comparison with controls on thiamine-supplemented diets, and with considerable variation from animal to animal. The glycogen content was considerably increased in animals first deficient then receiving thiamine and restricted calories, in comparison with the group continuously thiamine deficient. It was excessively increased in animals in a recovery stage on food ad libitum, for these livers showed a glycogen as well as a fat content beyond that of control animals continuously on a thiamine-supplemented basal diet. The pancreas was reduced in size in deficient animals, and the zymogen content appeared greatly decreased, except in the peri-insular zone. This was well seen in sections stained by Bowie's pepsinogen method (*32*) (fig. 12) and is in agreement with the observation of Ogata as reported by Jackscon (*33*) in pigeons. A similar peri-insular zone can usually be seen in normal animals, but it is less prominent. In recovery stages with

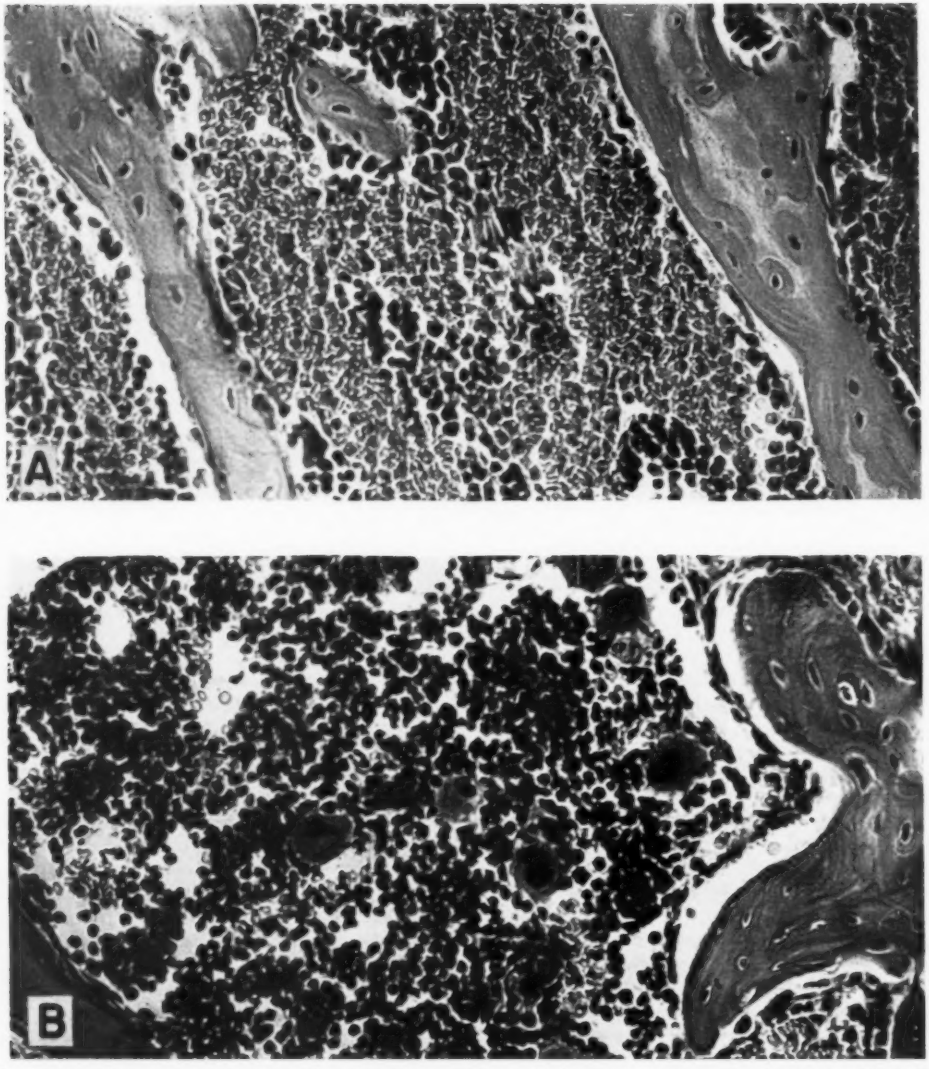

FIGURE 10.—Bone marrow. *A*, Atrophy and apparent congestion; *B*, Marrow from a control mouse. Hematoxylin-and-eosin stain. ×350.

thiamine-supplement and food ad libitum the acinar tissue of the pancreas was often spread apart by adipose cells. The skin showed an atrophy of sebaceous glands, with reduction in sudanophilic material.

The gastrointestinal mucosa of deficient animals showed engorgement of vessels and a reduced number of mitoses in the mucosal epithelium in comparison with the abundant mitoses in animals in recovery stages on food ad libitum. A small number of stomachs stained for pepsinogen by the Bowie method (*32*) showed a possible reduction in this material, but an exact estimation could not be made.

A frequent finding in the seriously deficient group was hemorrhage into the stomach or intestine, occurring 12 times in 50 animals. These gastrointestinal hemorrhages were observed only 6 times in animals with brain-stem hemorrhages, so there was no close correlation to the condition in the brain, although an increased tendency to hemorrhage at both sites was evident in thiamine-deficient animals.

A number of adrenals from each group was

stained for fat by the sudan III method. The amount of sudanophilic material was greatly reduced in the deficient animals and often was seen only in the zona glomerulosa. With thiamine and calorie restriction, fat became slightly more abundant and appeared to be evenly distributed in both the zona glomerulosa and zona fasciculata. In the pair-fed animals, the adrenal fat appeared to be slightly more reduced in the thiamine-deficient mice. On recovery of animals with un-

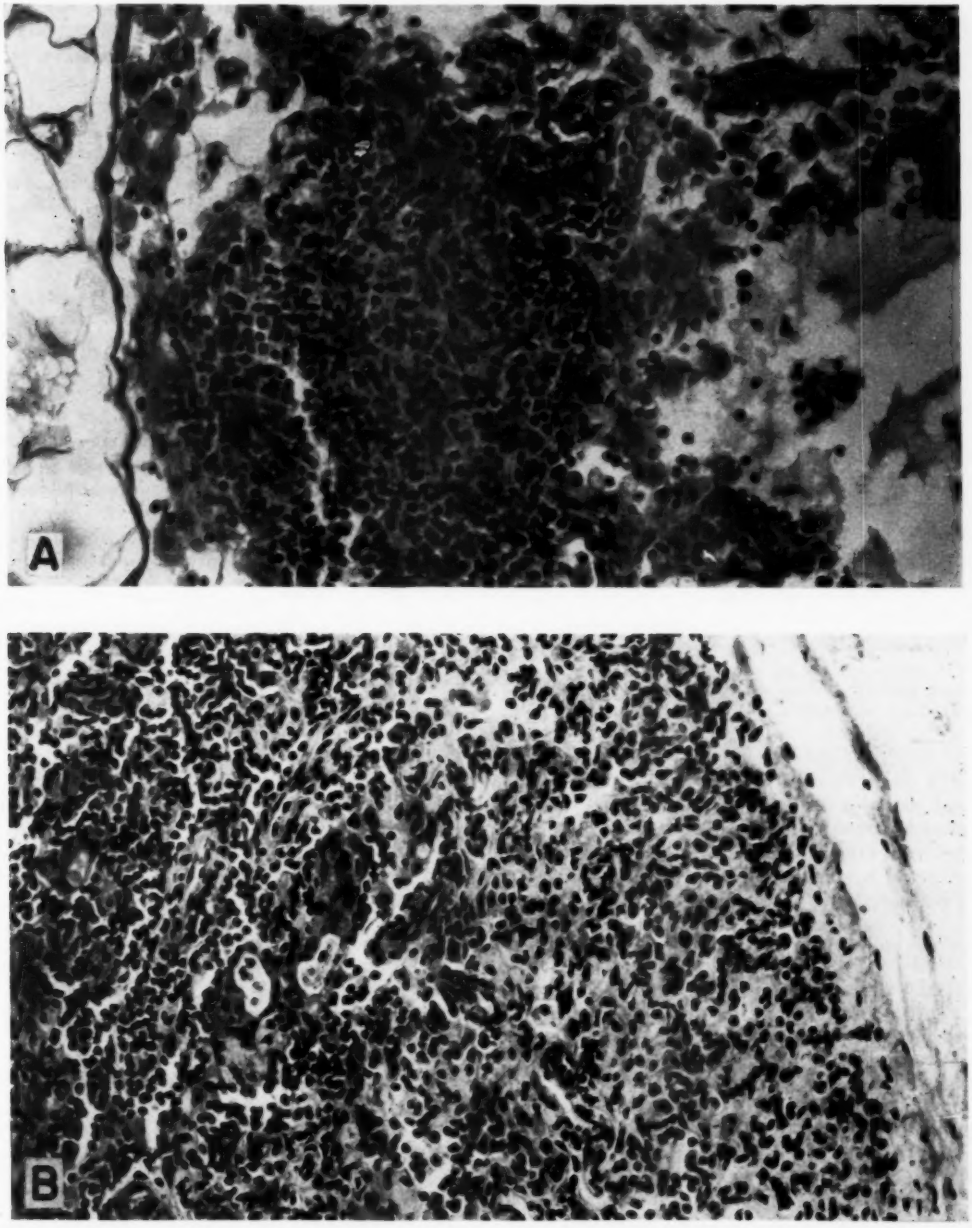

FIGURE 11.—Atrophy of lymphatic tissues. *A*, Lymph node showing reduction in lymphocytes and dilated sinuses; *B*, Thymus showing extreme reduction in lymphocytes and apparent increase in thymus reticulum. Hematoxylin-and-eosin stain. ×350.

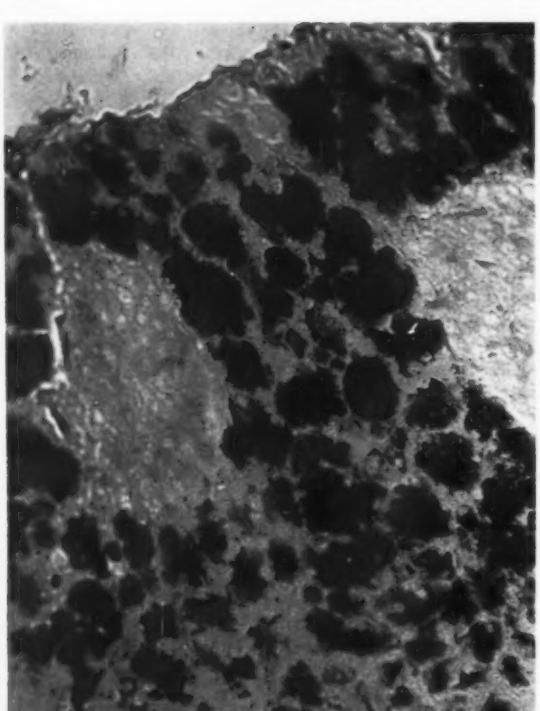

FIGURE 12.—Pancreas. Cells adjacent to islets contain pepsinogen granules, which are reduced in more distant cells. Bowie's pepsinogen stain. × 350.

restricted food, fat appeared to increase most rapidly in the zona fasciculata, and relatively little was found in the zona glomerulosa. This observation may be pertinent to the recent suggestion of Deane and Greep (*34*) that the "escalator" theory of cell migration in the adrenal cortex needs to be modified.

DISCUSSION

This study was designed as a survey of the effect of prolonged partial thiamine deficiency in the mouse. It was hoped that some basic histologic picture might be found which would indicate a common mechanism in the great variety of lesions encountered. Such an underlying mechanism exists in vitamin C deficiency, in which a failure of intercellular cement substances develops. The present findings, however, offer no suggestion of a single factor that will explain the many different lesions found in these mice. The possibility nevertheless exists that there may be an underlying common basic condition modified by species differences and the varying degrees of deficiency.

The experimental results here reported are impaired somewhat by the lack of a satisfactory control, since the inanition which accompanies prolonged partial thiamine depletion may produce other deficiencies as well. The lesions observed in the mouse are similar to those described with thiamine deficiency in other animal species, but the qualitative and quantitative pronounced changes encountered in the strain C3H mouse appear to be noteworthy. This may be accounted for by the fact that the diet used was not entirely devoid of thiamine. The presence of traces of thiamine perhaps permitted survival for a sufficient length of time to permit the development of these extensive morphologic changes.

SUMMARY

Thiamine deficiency was produced in weanling strain C3H mice by feeding them for several weeks a diet containing 1 μg. of thiamine per gram of food. The animals developed a specific and fatal deficiency syndrome usually between the seventh and the ninth week. Certain morphologic changes occurred with such regularity in this deficiency syndrome, termed "chronic thiamine deficiency," that they may be considered characteristic for this type of deficiency.

There was a general decrease in cell volume, which affected the cytoplasm more noticeably than the nucleus; and since the vascular bed did not seem to be reduced, many organs and tissues of the deficient mice appeared hyperemic.

A hemorrhagic lesion occurred in the thiamine-deficient animals in the midbrain at about the point of junction of the pons and medulla. Histologic changes were not observed in other parts of the nervous system.

The reproductive system showed extreme atrophy, with reduced formation of corpora lutea in the ovary, and reduced to absent spermatogenesis in the testes. A similar histologic picture in the ovary was produced by food restriction alone, but the change in the testis was seen only in thiamine-deficient animals.

A lesion of the skeletal muscles occurred frequently in thiamine-deficient animals and occasionally in pair-fed control animals receiving the same quantity of thiamine-containing food as that consumed by the deficient animals. This muscle lesion appeared grossly as silvery streaks, while microscopically it appeared as a finely granular swollen area in the muscle fiber, surrounded by cells with indistinct peripheral margins which were often coherent. The muscles of the tongue, colon, and myocardium occasionally showed similar degenerative areas.

The hematopoietic and lymphatic systems showed extreme atrophy, the greatest reduction occurring in cells of the lymphocytic series. These changes were not specific for this type of thiamine deficiency because similar changes were observed in animals receiving thiamine but restricted to the same quantity of food as that consumed voluntarily by the deficient animals.

The liver of deficient mice showed an absence of fat and reduced glycogen. During recovery, following thiamine alimentation with food ad libitum, fat and glycogen appeared in the liver in excessive amounts.

An increase in mitoses was observed in the gastrointestinal mucosa, the spleen, and lymph nodes during recovery stages.

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EFFECT OF GONADECTOMY AND ANDRENALECTOMY ON THE APPEARANCE AND INCIDENCE OF SPONTANEOUS LYMPHOID LEUKEMIA IN C58 MICE[1,2]

By L. W. Law,[3] with the technical assistance of Lester E. Bunker, Jr., and Betty-Ann Norris, *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*

INTRODUCTION

The significant sex differences in the incidence of leukemia in the human being and in some strains of mice have not as yet been explained adequately. In the human being the incidence of leukemia is significantly higher in the male (70 percent of the cases) (*1*) whereas in some strains of mice the incidence is significantly higher among females (*2, 3*); and it is found that females of certain strains are more susceptible to the induction of leukemia with estrogens (*4*). In several leukemic strains of mice, however, the influence of endogenous estrogen was not indicated, e. g., strain dba (*5*), strain F (*6*), and strain C58 (*7*).

A possible explanation for sex differences in the incidences of leukemia may be the opposing effects of the ovarian and testicular hormones. Several experiments designed to test this relationship have given contradictory results. Murphy (*3*) found that following gonadectomy in the RIL strain of mice, the male sex hormone apparently produced a definite inhibitory effect. Castrated males had a significantly higher incidence of leukemia, and the disease appeared earlier than in intact males; and ovariectomized females treated with testosterone exhibited a statistically significant lower incidence of leukemia than castrated or intact females. Ovariectomy had no effect on the incidence of leukemia. In line with Murphy's explanation, Silberberg and Silberberg (*8*) found that there was a definite inhibition of estrogen-induced leukemia in D strain mice castrated early in life. The presence of the testicle in sexually immature males apparently inhibited the development of leukemia. On the other hand, McEndy, Boon, and Furth (*9*) found that ovariectomy decreased the incidence of leukemia in the Ak strain, whereas the effect of orchidectomy was insignificant. A possible explanation for the difference in results obtained by Murphy and McEndy, Boon, and Furth in the closely related leukemic Ak and RIL strains of mice may be the age at which castration was effected (*3*). Gardner et al. (*4*) found that the simultaneous administration of testosterone propionate with estrogen reduced the incidence of lymphoid tumors in seven inbred strains of mice to approximately that of untreated controls. Pybus and Miller, as reported by Grüneberg (*10*), showed also that ovariectomy delayed the onset of the disease in females of one of their strains of mice. Data are not available regarding the age factor in this experiment. On the other hand, in strain F mice in which there is no sex difference in the incidence of leukemia, gonadectomy in sexually immature mice did not influence the spontaneous development of leukemia (*6*).

EXPERIMENTAL PROCEDURE

Effect of Gonadectomy

In order to determine the role of sex hormones in the high leukemia strain C58 mice,[4] gonadectomies were performed at 4 to 5 weeks of age and mice observed for leukemia. In order to reduce to a minimum the effect of extrinsic factors, animals of the seventy-fourth and seventy-fifth inbred generations were gonadectomized over a 2-month period. Litters were divided equally so that intact control animals were sibs of the experimental groups.

Observations were made daily throughout the experiments, and diagnosis of leukemia was based upon histologic findings in infiltrated tissues and blood smears. In the C58 strain usually a single subcutaneous lymph node is involved initially. At this time immature leukocytes are found in the blood, and the animal develops a generalized, systemic disease with a progressive leukocytosis and increase in absolute number of immature lymphocytes (*7*).

Orchidectomy significantly increased the incidence of lymphoid leukemias (difference of means, 32.3 ± 10.7 percent). Although leukemias appeared much earlier in the orchidectomized group,[5] the mean age at appearance of symptoms was the same. The mean-percentage difference of leukemia incidence in the ovariectomized and intact control groups was not statistically significant. Although leukemias appeared somewhat earlier in the experimental group (7.5 ± 0.88 months as against 8.8 ± 0.73 months in the intact females), the difference of the means (1.3 ± 1.14 months) is not significant (table 1).

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[2] Work supported under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council. This experiment was terminated by fire at Bar Harbor when the gonadectomized mice (nonleukemic, living) were 12 to 14 months of age and the adrenalectomized mice were 8 to 10 months of age.

[3] Present address, National Cancer Institute, Bethesda, Md.

[4] The C58 strain of E. C. MacDowell, Carnegie Institution of Washington, Cold Spring Harbor, N. Y. The incidence of leukemia in our breeding colony was found to be 89.4 percent with a slightly higher incidence in females. The majority of leukemias have been found to be lymphoid in origin.

[5] Aside from two leukemias which appeared at 4 months of age in hypophysectomized mice (unpublished data), we have not observed leukemias occurring spontaneously in our breeding colony before 6 months of age.

Effect of Adrenalectomy

Data relating to the role of the adrenals in spontaneous or induced leukemia are scarce, although it has been reported that a significant part is played by the adrenals in susceptibility to a transplantable leukemia in the rat (*12, 13*). In view of the fact that adrenal cortical secretions have been found to produce definite palliative effects in terminal cases of lymphoid leukemia (*14*), the effect of adrenalectomy on the incidence and appearance of the spontaneous disease in the C58 strain was determined. Both adrenals were extirpated along with perirenal fat following nembutal anesthesia in mice at 4 to 5 weeks of age. Mice were maintained on 1 percent NaCl. Mortality was high at 2 and 3 months of age. Of 95 animals which survived the operation, only 55 survived 6 months or longer (time of appearance of first leukemia). Regeneration of adrenal tissue was observed in only 2 cases at autopsy.

Table 2 shows that there was a statistically significant increase in the incidence in the adrenalectomized series at the end of the 9-month period. Although 45 percent of the leukemias had appeared in the experimental group at the end of the 7-month period as compared with 19 percent in the control group, the mean age of appearance was not significantly earlier in the experimental group. The symptoms and course of the disease in adrenalectomized mice were similar to that in intact mice.

It must be borne in mind that the results reported herein are only indicative, since the experiments were terminated before the complete expression of the leukemic tendency in the experimental and control groups could be ascertained.

SUMMARY

Orchidectomy of immature males of strain C58 significantly increased the incidence of spontaneous lymphoid leukemia. In this strain of mice, ovariectomy had no apparent effect on the incidence of leukemia.

Adrenalectomy of immature male and female mice of the C58 strain significantly increased the incidence of spontaneous lymphoid leukemia.

Table 1.—*Effect of gonadectomy on incidence and appearance of spontaneous lymphoid leukemias in the C58 strain of mice*

Group	Number of mice	Mice with leukemias		Appearance of leukemia by month										Age of mice at appearance of leukemia	Nonleukemic mice at end of experiment[1]
				5	6	7	8	9	10	11	12	13	14		
		No.	*Pct.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*Months*	*Number*
Orchidectomized [2]	42	32	[3] 76.2	3	2	5	9	3	5	2	1	1	1	8.5	5
Ovariectomized	37	24	[3] 64.9	6	2	3	7	2	2	2	.....	.....	.....	7.5	13
Control males	41	18	[3] 43.9	.....	1	5	5	2	2	1	1	1	.....	8.6	17
Control females	45	27	[3] 60.0	.....	3	2	9	3	5	3	2	.....	.....	8.8	15

Difference of means, orchidectomized and control, 32.3±10.7 percent. $P<0.01$.

[1] Nonleukemic animals were from 12 to 14 months of age when experiment was terminated.

[2] Pedigreed mice of the seventy-fourth and seventy-fifth inbred generations. Controls are sibs to experimental groups.

[3] The percentages of mice dying from leukemia are based upon total number of mice surviving beyond the time of appearance of the first leukemia. Adventitious deaths within the period observed are small and do not affect the significance of the results.

Table 2.—*Effect of adrenalectomy on incidence and appearance of spontaneous lymphoid leukemias in the C58 strain of mice*

Group	Number of mice	Mice with leukemias		Appearance of leukemia by month							Age of mice at appearance of leukemia	Nonleukemic mice at end of experiment [1]
				5	6	7	8	9	10	11		
		Number	*Percent*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Months*	*Number*
Adrenalectomized males and females [2]	[3] 55	27	[3] 49.1	.....	6	6	5	10	.....	.....	7.7	27
Control males and females	65	16	24.6	.....	.....	3	9	4	.....	.....	8.4	45

Difference of means, adrenalectomized and control mice 24.5±8.7 percent. $P<0.01$.

[1] Nonleukemic animals were from 8 to 10 months of age when experiment was terminated.

[2] Pedigreed mice of seventy-fourth and seventy-fifth inbred generations. Control mice are sibs to experimental group.

[3] Since there was no sex difference in response to adrenalectomy, males and females are combined.

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THIRD CUBAN CANCER CONGRESS

The Third Cuban Cancer Congress under the auspices of the Governor of the Republic, the Cuban Society of Cancer, and the League Against Cancer will be held in Havana on May 2–7, 1948, instead of February 23–28, 1948, as previously reported. Inquiries should be directed to Comite Organizador, Oficinas de la Liga contra Cancer, Calle F esq. a 29, Vedado, Habana, Cuba.

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